ABSTRACT

Effects of Chiral Contaminants to Aquatic Organisms: Pharmaceuticals as Model Compounds for Enantiomer Specific Ecological Hazard Assessment

Jacob K. Stanley, Ph.D.

Mentor: Bryan W. Brooks, Ph.D.

In the present study, enantiospecific effects of chiral contaminants were explored using two chiral pharmaceutical contaminants as model compounds. These compounds are the selective serotonin reuptake inhibitor antidepressant fluoxetine and the β-adrenergic receptor blocking agent propranolol. An aquatic invertebrate, *Daphnia magna*, and an aquatic vertebrate, *Pimephales promelas*, were used as model organisms. In addition to commonly used standardized bioassay endpoints, effects of these compounds were also assessed using nontraditional sublethal endpoints that were specifically chosen to target the known modes of action of the model pharmaceuticals. These include *D. magna* heart rate and grazing rate and *P. promelas* feeding rate, swimming performance, and swimming behavior. Known enantiospecific differences in activity of propranolol and fluoxetine in mammals were compared with enantiospecific differences in their toxicity to aquatic organisms. Results indicate that mammalian pharmacology data on enantiospecific effects are more predictive of enantiospecific toxicity in aquatic vertebrates than invertebrates for the two drugs tested. The results

presented here also demonstrate that mode-of-action-targeted endpoints should be considered for pharmaceuticals as they can be more sensitive than traditional endpoints, show enantiospecific and sex-specific effects, and provide information on highly ecologically relevant biological processes such as feeding. A summary of the current regulatory provisions for chiral contaminants is made along with the author's recommendations for the improvement of the assessment of environmental risk for chiral contaminants.

Considering Enantiospecific Effects of Chiral Pharmaceutical Contaminants to Model Aquatic Organisms: Effects on Behavioral, Physiological, and Traditional Toxicity Testing Endpoints

by

Jacob K. Stanley, B.S., M.S.

A Dissertation

Approved by the Department of Biology

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Robert D. Doyle, Ph.D., Chairperson

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Approved by the Dissertation Committee

_____

Bryan W. Brooks, Ph.D., Chairperson

_____

Jason B. Belden, Ph.D.

_____

Robert D. Doyle, Ph.D.

_____

Ryan S. King, Ph.D.

_____

C. Kevin Chambliss, Ph.D.

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J. Larry Lyon, Ph.D., Dean

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ANOVA	Analysis of Variance
APHA	American Public Health Association
AWWA	American Water Works Association
CCD	charge-coupled device
DDT	dichlorodiphenyltrichloroethane
EC50	50% effect concentration
EHR	enantiomer hazard ratio
ELISA	enzyme-linked immunosorbent assay
ER	enantiomer ratio
ERA	ecological risk assessment
EROD	7-ethoxyresorufin-*O*-deethylase
$F_{SS}PC$	fish steady state plasma concentration
h	hour
HI	hazard index
HPLC	high performance liquid chromatography
K_{ow}	octanol-water partitioning coefficient
LC50	50% lethal concentration
LC-MS/MS	liquid chromatograph-tandem mass spectrometer
LOEC	lowest observed effect concentration
MEC	measured environmental concentration
MFO	mixed function oxygenase
MS/MS	tandem mass spectrometer
m/*z*	mass/charge
NOEC	no observed effect concentration
NPY	neuropeptide Y
OECD	Organization for Economic Co-operation and Development
PEC	predicted environmental concentration
PCB	polychlorinated biphenyls
PVC	polyvinyl chloride
QSAR	quantitative structure-activity relationship
rac	racemic
RHW	reconstituted hard water
s	second
SBC	screening benchmark concentration
SEC	screening exposure concentration
SSRI	selective serotonin reuptake inhibitor
TBC	toxicological benchmark concentration
TU	toxic unit
U_{crit}	critical swimming speed
USA	United States of America

U.S. EPA	United States Environmental Protection Agency
U.S. FDA	Unites States Food and Drug Administration
WEF	Water Environment Foundation

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DEDICATION

To Kristen,

Thank you for your unwavering love and support

CHAPTER ONE

Introduction, Background, and Chapter Overview

Many environmental contaminants including pharmaceuticals, pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and musk fragrances are chiral and are often distributed as racemic mixtures. A chiral molecule has two or more stereoisomers that are non-superimposable mirror images of each other, known as enantiomers. A racemic mixture is a 1:1 mixture of enantiomers. Enantiomers have identical molecular formulae and identical physical and chemical properties; however, they can differ markedly in potency, toxicity, bioavailability, and environmental fate due to the stereospecific nature of biological receptors (Stanley et al. 2006, McConathy and Owens 2003, Ali and Aboul-Enein 2004).

While enantiospecific differences in fate and effects for chiral contaminants have recently been increasingly recognized, these differences are often largely ignored in the fate and effect studies leading to assessments of environmental risk for such compounds. That is, mixtures of enantiomers are treated as one compound in ecotoxicity testing and environmental monitoring or analytical quantitation. This neglect of stereospecific differences between enantiomers introduces unnecessary uncertainty into environmental risk assessments for chiral compounds.

Of the limited published studies that have investigated enantiospecific differences in environmental contaminants, many more studies have investigated enantiospecific fate than effects to nontarget organisms. Also, the effect data that is available almost exclusively focuses on acute effects to invertebrate species and do not consider often

more environmentally relevant sublethal effects. Because of the lack of published enantiospecific effect data, in the present study, I chose to select two model compounds to investigate sublethal effects of chiral contaminants. I chose to use known chiral pharmaceutical contaminants as models because, unlike other chiral contaminants, there is often data available on enantiospecific differences in effects for these compounds due to the drug development and registration process. Also, mammalian pharmacodynamic, pharmacokinetic, and toxicological information generated during drug development may be leveraged to focus ecotoxicological research on endpoints and compounds with a greater potential to sublethally impact aquatic ecosystems (Seiler et al. 2002, Huggett et al. 2003a).

Summary of Chapter Contents

This document is divided into five chapters. Chapter two describes experimentation conducted with the first model chiral contaminant chosen, the non-selective β-adrenergic receptor blocking agent heart medication propranolol. I chose to begin studies with this compound because of the approximately 100-fold difference in β-adrenergic receptor blocking activity observed in mammals between the two enantiomers of this widely used and environmentally detected pharmaceutical (Howe and Shanks 1966, Barrett and Cullum 1968, Huggett et al. 2003a). Also, experiments with the racemic form of this drug had previously been shown to affect the sublethal physiological endpoint heart rate in an aquatic invertebrate (Dzialowski et al. 2006). The ability to compare the magnitude of enantiospecific differences in effects of propranolol on this clearly ecologically relevant endpoint in mammals with aquatic invertebrates was another primary reason this drug was chosen. A primary focus of this dissertation is the

development and use of ecotoxicological endpoints that are specifically selected to assess effects on non-traditional endpoints that are targeted towards suspected modes of toxic action in aquatic organisms. Model aquatic species used in this and other studies described herein were the aquatic invertebrate "water flea", *Daphnia magna*, and the aquatic vertebrate teleost, the fathead minnow (*Pimephales promelas*). Other than the assessment of acute exposures of propranolol on the invertebrate heart rate endpoint, a battery of acute and chronic ecotoxicity studies were also performed in order to investigate enantiospecific differences in effect of propranolol exposure on commonly measured ecotoxicological endpoints such as survival, growth, and reproduction.

Chapter three describes initial studies performed with the second model chiral compound used, the selective serotonin reuptake inhibitor antidepressant pharmaceutical fluoxetine. Fluoxetine was chosen because of the wide array of physiological and behavioral processes controlled or influenced by the serotonergic system, the target of this pharmaceutical, in vertebrate and invertebrate organisms (Fong 2001). The two enantiomers of fluoxetine are different than those of propranolol in that they are very similar in their ability to block serotonin reuptake at the synapse. However, an approximately 20-fold difference in serotonin reuptake inhibition exists between the enantiomers of the primary active metabolite of fluoxetine, norfluoxetine, making fluoxetine another excellent compound to use to examine enantiospecific differences in effects to non-target organisms. Again, acute and chronic ecotoxicological bioassays were performed with the two enantiomers of this drug using the model aquatic organisms *D. magna* and *P. promelas* in order to assess enantiospecific differences in effects of this chiral compound. The mode of action of this pharmaceutical compound also provided

the opportunity to examine enantiospecific effects to another highly-ecologically relevant mode-of-action-targeted sublethal endpoint in *D. magna* and *P. promelas*, feeding behavior.

Because *S*-fluoxetine was found to be significantly more toxic to juvenile fathead minnows in the above-mentioned study, I decided to more thoroughly investigate the effects of this enantiomer on other sublethal endpoints in fish that are influenced by serotonergic control in longer-term studies. Long-term chronic exposures of pharmaceutical contaminants are highly environmentally relevant since the rates of introduction of these compounds to the environment via municipal effluents often exceed their half-lives in effluent dominated receiving systems, a phenomenon labeled "pseudopersistence" in the literature (Brooks et al. 2006). This work is covered in chapter four. In order to examine effects on fish reproduction as well as age and sex-specific differences in toxicity, sexually mature adult fish were used in this study, contrary to the juvenile fish used in the study described in chapter three. In addition to reproductive endpoints, swimming performance and behavior as well as feeding rate were assessed in the adult fish. Also, internal dose of fluoxetine was quantitated by measuring plasma fluoxetine concentration in individual fish.

The final chapter of this dissertation, chapter five, takes a broader look at the issue of chiral contaminants in the environment. Existing regulatory guidance on chiral pharmaceuticals and pesticides is discussed and critiqued. Also, the aspects of stereochemistry that present challenges to the assessment of environmental risk via complicating measures of environmental fate and effects for chiral compounds are

discussed. Finally, recommendations are made to improve the way stereochemistry is considered in the environmental risk assessments of chiral compounds.

CHAPTER TWO

Enantiospecific toxicity of the β-blocker propranolol to *Daphnia magna* and *Pimephales promelas*

Introduction

Many xenobiotics, including several known environmental pharmaceutical contaminants, are chiral molecules. A chiral molecule has two or more stereoisomers that are non-superimposable mirror images of each other, known as enantiomers (Figure 2.1). Enantiomers have identical molecular and structural formulae, but differ in their

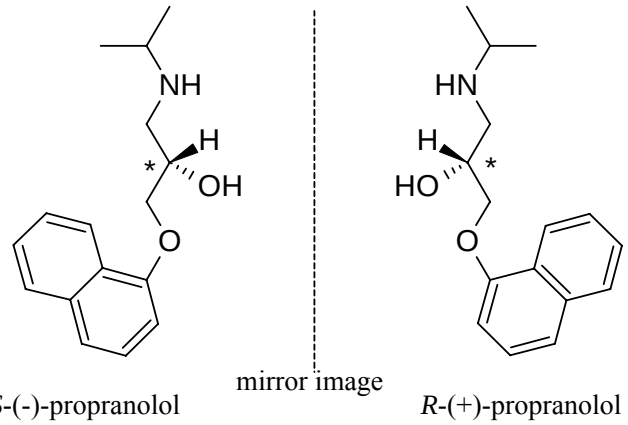

Figure 2.1 Structure and configuration of the *S*-(-)- and *R*-(+)-enantiomers of propranolol (stars indicate chiral carbons)

spatial configuration. A 1:1 mixture of enantiomers is known as a racemic mixture. A few studies have considered chiral environmental contaminants and enantiospecific toxicity of organochlorine and organophosphorus pesticides and other agrochemicals (Hegeman and Laane 2002), but ecotoxicity data regarding enantiospecific toxicity of chiral pharmaceutical contaminants are lacking. Examples of chiral pharmaceuticals that

are distributed as racemates and have been measured in municipal effluents and surface waters include propranolol (β-blocker), fluoxetine (selective serotonin reuptake inhibitor), and ibuprofen (analgesic) (Ternes 1998, Kolpin et al. 2002, Huggett et al. 2003a). To perform a thorough risk assessment for these chiral pharmaceutical contaminants in aquatic systems, it is essential to determine the potential of each enantiomer to reach a site of toxic action and exert a toxic effect on aquatic organisms. However, I am not aware of any enantiospecific toxicity or fate data in the literature for chiral pharmaceutical contaminants.

Other than their differential rotation of the plane of polarized light, the different enantiomers of a chiral compound exhibit identical physical and chemical properties in a symmetrical environment (Kallenborn and Hühnerfuss 2001). However, when these compounds are present in an asymmetrical biological environment, such as the highly specific binding sites of many cellular receptors, enantiospecific differences in biological activity can occur (Kallenborn and Hühnerfuss 2001, Mathison et al. 1989). Such differences occur because of the differential spatial orientation of the functional groups of each enantiomer with the receptor complex or because the two enantiomers arrive at the receptor at different concentrations because of enantioselective processes such as membrane permeability or metabolism (Mathison et al. 1989). Enantiomers also may differ significantly in their rate of uptake and excretion, affinity for plasma proteins, structure of metabolites, potency, toxicity, and bioavailability (McConathy and Owens 2003, Ali and Aboul-Enein 2004). The different enantiomers of a chiral pharmaceutical can have such distinct pharmacological properties that they should be considered two distinct drugs until proven otherwise (McConathy and Owens 2003).

A trend exists towards the increased development of single-enantiomer drugs because they can potentially have simpler and more selective pharmacologic profiles, improved therapeutic indices, simpler pharmacokinetics, and decreased drug interactions (McConathy and Owens 2003). Hutt (2002) estimated that approximately 50% of commercially available pharmaceuticals are chiral compounds, and Tran et al. (2004) stated that 61 of the 528 chiral synthetic drugs are marketed as single enantiomers while the other 467 are sold as racemates. Enantiospecific biological effects are well known by the pharmaceutical industry. Many of the most widely distributed drugs are marketed as single enantiomers to avoid possible adverse side effects associated with the undesirable enantiomer or to make use of the major advantages in using stereochemically pure drugs: Reduction of the total administered dose, enhanced therapeutic window, reduction of intersubject variability, and more precise estimation of dose-response relationships (Caner et al. 2004). Worldwide sales of chiral drugs in the single-enantiomer form rose from 27% of all distributed drugs in 1996 to 39% in 2002 (Caner et al. 2004).

Organisms degrade chiral compounds via stereospecific enzymatic processes that can result in a change in the ratio of the concentrations of each of the enantiomers in the environment, known as the enantiomer ratio (ER) (Hegeman and Laane 2002). A change in the measured environmental ERs from that of the original ER of the contaminant can be used as a tracer in environmental fate studies (Hegeman and Laane 2002). For example, a pharmaceutical compound that is distributed as a racemate may have an ER significantly different than 1:1 when the concentrations of each enantiomer are measured in the environment (Fono and Sedlak 2004). Fono and Sedlak (2004) used environmental ERs of a β-blocker to discriminate between treated wastewater and untreated sewage

from combined sewer overflows as sources of contamination by wastewater-derived contaminants. Enantiospecific environmental degradation and/or enantiospecific metabolism of racemic mixtures of chiral contaminants also may lead to measured ERs that differ from 1:1 in the tissues of exposed organisms (Hummert et al. 1995).

Propranolol ($C_{16}H_{21}NO_2$; mol wt, 259.3), a non-selective β-adrenergic receptor blocking agent, is widely prescribed as a racemate for the treatment of angina and hypertension and is also used as a migraine prophylactic and to control symptoms of anxiety. Propranolol is a pure β_1- and β_2-receptor antagonist (Mehvar and Brocks 2001). It also is a weak antagonist of β_3 receptors (Tsujii and Bray 1998), which are present in fish (Nickerson et al. 2003). Propranolol inhibits the action of adrenergic agents and reduces heart rate and the force with which the heart muscle contracts (Tran et al. 2004). Propranolol has been detected in municipal effluents and surface waters with levels ranging from the ng/L to the low µg/L range (Ternes 1998, Huggett et al. 2003a, Thomas and Hilton 2004). Propranolol is distributed as a racemic mixture (*rac*-propranolol hydrochloride) of *S*-(-)-propranolol hydrochloride and *R*-(+)-propranolol hydrochloride. The *S*-enantiomer is by far the most active form in β-adrenergic blocking activity (up to 100-fold difference) in mammals (Howe and Shanks 1966, Barrett and Cullum 1968). The *R*-enantiomer is responsible for propranolol's membrane stabilizing effect (Kim et al. 2003). Propranolol also acts as a serotonin-receptor antagonist in a stereoselective manner, with *S*-propranolol being the more potent enantiomer (Alexander and Wood 1987). The *S* to *R* concentration ratio of each enantiomer that is excreted in human urine after dosage with the racemic form is approximately 1.3 to 1.4 (Pham-Huy et al. 1995).

Because of the normally trace-level environmental concentrations of pharmaceutical contaminants (usually in the ng/L range) and their often-specific modes of action, investigations into physiological responses of exposed aquatic biota may provide information regarding the energetic and potential ecological consequences of exposure to these contaminants. This is critical because traditional whole-effluent toxicity testing endpoints may not be sensitive enough to characterize adequately the risk associated with these chemicals (Brooks et al. 2003a). Whereas many studies regarding the occurrence of pharmaceutical contaminants in the environment exist, relatively few studies have examined sublethal responses of aquatic biota to these contaminants. Until biochemical, physiological, and life history consequences of low-level exposure to aquatic pharmaceuticals can be determined, thorough assessments of ecological risk will be challenging. The purposes of the present study were to investigate enantiospecific toxicity of propranolol to *Daphnia magna* and *Pimephales promelas* and examine the effects of acute propranolol exposure on *D. magna* heart rate. Our research hypotheses were that the *S*-enantiomer would be more toxic to *D. magna* and *P. promelas* and exhibit a greater dose-dependent effect on *D. magna* heart rate than its antipode.

Materials and Methods

All toxicity tests were carried out using reconstituted hard water made according to U.S. Environmental Protection Agency (U.S. EPA) methods (U.S. EPA 2002a). Reconstituted hard water was also used as control water for all tests. Alkalinity (mg/L as $CaCO_3$) and hardness (mg/L as $CaCO_3$) of reconstituted hard water were measured by colorimetric titration before acute and chronic test initiation and at renewal of chronic tests according to standard methods (APHA, AWWA, WEF 1998). Specific conductance

(µs/cm), pH, and dissolved oxygen (mg/L) of reconstituted hard water were also measured at these times using a multiprobe. All culturing and toxicity tests were carried out at a temperature of 25 ± 1 °C and a 16:8 hour light-dark cycle. The *rac*-(±)-propranolol hydrochloride, *S*-(-)-propranolol hydrochloride, and *R*-(+)-propranolol hydrochloride were purchased from Sigma (St. Louis, MO, USA). Both propranolol enantiomers and the racemic mixture were introduced as propranolol HCl; however, all values reported in this study are for propranolol and not its hydrochloride salt. A solvent carrier was not required to dissolve propranolol HCl into the reconstituted hard water.

D. magna *48-h Acute Toxicity Tests*

Two *D. magna* 48-h acute exposures were performed following U.S. EPA test method 2021.0 (U.S. EPA 2002a). Daphnids younger than 24 h were used to initiate these tests. Test containers were 30-ml disposable plastic cups with a test volume of 25 ml. The experimental design consisted of five *D. magna* per test chamber, and four test chambers per treatment level. Two hours prior to test initiation, *D. magna* were fed a mixture of *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) and cereal grass media (ScholAR™ Chemistry, Avon, NY, USA) in RHW (U.S. EPA 2002a, Hemming et al. 2002). Nominal treatment levels of propranolol for the *D. magna* exposures were: racemic mixture and *S*-enantiomer: 4000, 2000, 1000, 500, and 250 µg/L; *R*-enantiomer: 4800, 2400, 1200, 600, and 300 µg/L. Immobilization was the lone endpoint assessed in these exposures.

D. magna *21-d Chronic Toxicity Test*

A 21-d *D. magna* chronic toxicity test was carried out following a procedure adapted from the Organization for Economic Co-operation and Development (OECD 1998) and the U.S. EPA (1996). Daphnids younger than 24 h were used to initiate this test. Test containers were 30-ml disposable plastic cups with a test volume of 30 ml. This was a static renewal test. The experimental design consisted of one daphnid per test chamber, and 10 test chambers per treatment level. *Daphnia magna* were fed 0.6 ml/d of a mixture of *P. subcapitata* and cereal grass media (U.S. EPA 2002a, Hemming et al. 2002). Endpoints were *D. magna* immobilization and reproduction as young per surviving organism. Renewal of test media was carried out every other day. Nominal treatment levels for this exposure were 800, 400, 200, 100, and 50 µg/L for both the *R*- and *S*-enantiomers and the racemic mixture.

D. magna *Heart Rate Study*

The transparency and relatively large size of *D. magna* makes this species a good model for studies of physiological effects of anthropogenic contaminants on aquatic invertebrates (Villegas-Navarro et al. 2003, Dzialowski et al. 2006). A *D. magna* heart rate study was carried out according to methods found in (Dzialowski et al. 2006). Five-day-old *D. magna* were exposed for 30 minutes to nominal concentrations of 742.5, 1485, and 2970 µg/L of the *R*- and *S*-enantiomers of propranolol. These treatment levels were chosen because they represent approximately half, equal, and twice the average concentrations producing a 50% effect (EC50) in the 48-h acute immobilization test for *R*- and *S*-propranolol. Each *D. magna* was exposed individually in a 30 ml disposable plastic cup with a test volume of 25 ml. Ten replicates were tested per treatment level.

At the end of each individual organism's 30 minute exposure period, the organism was gently transferred to a microscope slide in a drop of water using a large bore pipette. Excess water was quickly removed with a pipette to prevent the organism from moving out of the microscope's field of view. Finally, heart rate (heart beats minute^{-1}) was determined using a stereomicroscope. A three second digital video clip was taken at 30 frames per second using the imaging software package Image Pro Plus 5.0® (Media Cybernetics, Silver Spring, MD, USA). All recordings were made within 30 s of the *D. magna* being placed on the slide to ensure that all organisms were exposed on the slide for the same period of time.

P. promelas *48-h Acute Toxicity Test*

Two 48-h acute *P. promelas* toxicity tests were performed following U.S. EPA test method 2000.0 (U.S. EPA 2002a). Test chambers were 600 ml glass beakers with a test solution volume of 250 ml. The experimental design consisted of ten *P. promelas* larvae per test chamber, and four test chambers per treatment level. *Pimephales promelas* larvae were fed newly hatched *Artemia* nauplii two hours before initiation of testing according to U.S. EPA methods (U.S. EPA 2002b). Nominal treatment levels for these experiments were 3.51, 1.75, 0.88, 0.44, and 0.22 mg/L for both the *R*- and *S*-enantiomers and the racemic mixture. Eight-day-old larvae were used to initiate this experiment. Survival was the lone endpoint measured in this exposure.

P. promelas *7-d Short-Term Chronic Toxicity Test*

A *P. promelas* 7-d larval survival and growth test was performed following U.S. EPA test method 1000.0 (U.S. EPA 2002b). This test was initiated with eight-day-old

larvae. Test chambers were 600 ml glass beakers with a test solution volume of 250 ml. The experimental design consisted of ten *P. promelas* larvae per test chamber, and four test chambers per treatment level. *Pimephales promelas* larvae were fed newly hatched *Artemia* nauplii daily according to U.S. EPA methods (U.S. EPA 2002b). Nominal treatment levels for this experiment were 1500, 750, 375, 187.5, and 93.8 µg/L. Endpoints measured in this exposure were survival and growth, measured as dry weight.

Liquid Chromatography/Mass Spectrometry Analysis

All exposure concentrations were verified analytically using a liquid chromatograph/mass spectrometer. Prior to analysis, all control, calibration, and toxicological samples were spiked with 100 ng of 10,11-dihydrocarbamazepine (Sigma, St. Louis, MO, USA) as an internal standard. Control samples consisted of reconstituted hard water spiked with internal standard only (hereafter referred to as a "blank"). Matrix-matched calibration samples were prepared by serial dilution of 1 mg/L or 10 mg/L propranolol stock solutions in reconstituted hard water, resulting in calibrators ranging from 0.5-700 µg/L. Fresh control and calibration samples were prepared for each set of toxicological samples to be analyzed.

All analyses were performed using a benchtop liquid chromatograph/mass spectrometer consisting of a Varian ProStar® high performance liquid chromatogrpahy system coupled to a Varian Model 1200L triple-quadrupole mass analyzer (Varian, Palo Alto, CA, USA). The analyzer was calibrated and tuned at the beginning of the analysis using a Varian Mass Calibration and Tuning Solution: Tetraalkylammonium Compounds (03-937281-01). Chromatographic separation of propranolol and 10,11-dihydrocarbamazepine was achieved using a 50 mm x 2.1 mm internal diameter

AquaSep® 5µm, 100 Å column (ES Industries, West Berlin, NJ, USA) with isocratic elution at a flow rate of 0.3 ml/minute. The mobile phase consisted of 75% 20 mM aqueous ammonium acetate containing 0.1% (weight/volume) formic acid and 25% acetonitrile. An autoinjector in partial loop mode was used to achieve a reproducible injection volume of 10µL for each run. The mass spectrometer was operated in positive electrospray ionization mode with single ion monitoring. Detector, needle, and shield voltages were set to 1.4 kV, 5.0 kV, and 0.6 kV, respectively. The molecular ions $[M+H^+]$ of target analytes were monitored at a mass/charge ratio of 260 for propranolol and 239 for the internal standard.

Analyte concentrations in both control and toxicological samples were determined using an internal standard calibration procedure. The response factor was calculated by dividing the peak area of the propranolol by the peak area of the internal standard. Calibration curves were prepared by plotting a linear regression ($r^2 \geq 0.998$) of the analyte/internal standard response factor versus analyte concentration for all calibrators analyzed. Calibration was monitored through the use of continuing calibration verification samples with an acceptability criterion of ± 20%. In a given run, one blank and one continuing calibration verification sample were interspersed between every five toxicological samples for quality assurance purposes. Reported analytical concentrations of propranolol in toxicological samples represent an average result for three sample injections plus or minus one standard deviation ($n = 3$). Measured propranolol concentrations were 113.9 ± 16.5% of nominal concentrations, on average, in all experiments.

Statistical Analyses

Statistical significance of response variables was determined at $\alpha = 0.05$ for all tests. The EC50 and 50% lethal concentration (LC50) values for *D. magna* and *P. promelas* acute tests, respectfully, were calculated using the probit method if possible; however, if assumptions of the probit method were not met, the Trimmed Spearman-Karber method was used. Proportional mortality data were arc sine (square root (y)) transformed prior to hypothesis testing. A Fisher's Exact Test was used for the immobilization endpoint in the *D. magna* 21-d chronic test. A Steel's Many-One Rank test was used to analyze *P. promelas* survival in the 7-d chronic test. Analyses of reproduction and growth endpoints for all tests were determined using parametric one-way analysis of variance (ANOVA) along with a Dunnett's multiple range test or a *t*-test with Bonferroni adjustment, as needed, according to (U.S. EPA 2002b). Analyses of the *D. magna* heart rate data were performed using a parametric one-way ANOVA along with a Dunnett's multiple range test.

Results

Mean (± one standard deviation) reconstituted hard water water quality parameters for all tests were as follows: pH = 7.6 (± 0.2), dissolved oxygen = 6.1 mg/L (± 1.3), specific conductance = 554.0 µs/cm (± 27.3), hardness = 162.4 mg/L as $CaCO_3$ (± 7.1), and alkalinity = 111.3 mg/L as $CaCO_3$ (± 5.6).

In this paper, all references to a treatment level refer to a nominal concentration of propranolol, where all effect endpoints (lowest observed effect concentration (LOEC), LC50, or EC50) refer to analytically measured concentrations of propranolol. Calculated average (n = 2) 48-h immobilization EC50s from the *D. magna* acute tests were 1.40,

1.57, and 1.67 mg/L for *S*-, *R*-, and *rac*-propranolol, respectively. Resulting EC50s from each individual acute *D. magna* exposure with associated 95% confidence intervals for each enantiomer treatment were: *S*-propranolol – 1.38 mg/L (1.00, 1.73) and 1.41 mg/L (1.17, 1.71); *R*-propranolol –1.53 mg/L (1.37, 1.71) and 1.61 mg/L (1.26, 2.00); *rac*-propranolol – 1.46 mg/L (1.23, 1.75) and 1.87 mg/L (1.56, 2.24). Immobilization LOECs for *R*-, *rac*-, and *S*-propranolol in the 21-d chronic study were 409.3, 843.7, and >869.0 µg/L, respectively. Significant increases in *D. magna* reproductive output (as mean neonates per surviving female) were observed for all treatments at the 50 µg/L treatment level, for *R*-and *rac*-propranolol at the 200 µg/L treatment level, and the 400 µg/L treatment level of *rac*-propranolol (Figure 2.2). The only significant decrease in reproduction in a treatment that was not significantly different from controls for immobilization was observed at the 800 µg/L treatment level of *S*-propranolol (measured concentration = 869.0 µg/L) (Figure 2.2).

Results from the acute (30 minute exposures) *D. magna* heart rate study (Figure 2.3) showed no enantiospecific differences in the effects of propranolol enantiomers on *D. magna* heart rate. However, significant reductions in heart rate were observed at the highest treatment level tested (2,970 µg/L) in both the *R*- and *S*-enantiomer treatments. Measured propranolol concentrations for these treatments were 2,612 and 2,621 µg/L, respectively. Calculated average (n = 2) LC50s from the *P. promelas* 48-h acute tests were 1.42, 1.69, and 1.21 mg/L for *S*-, *R*-, and *rac*-propranolol, respectively. Resulting LC50s from each individual acute *P. promelas* exposure with associated 95% confidence intervals for each enantiomer treatment were: *S*-propranolol – 1.11 mg/L (0.92, 1.29) and 1.72 mg/L (1.53, 1.94); *R*-propranolol –1.42 mg/L (1.19, 1.64) and 1.95 mg/L (1.74,

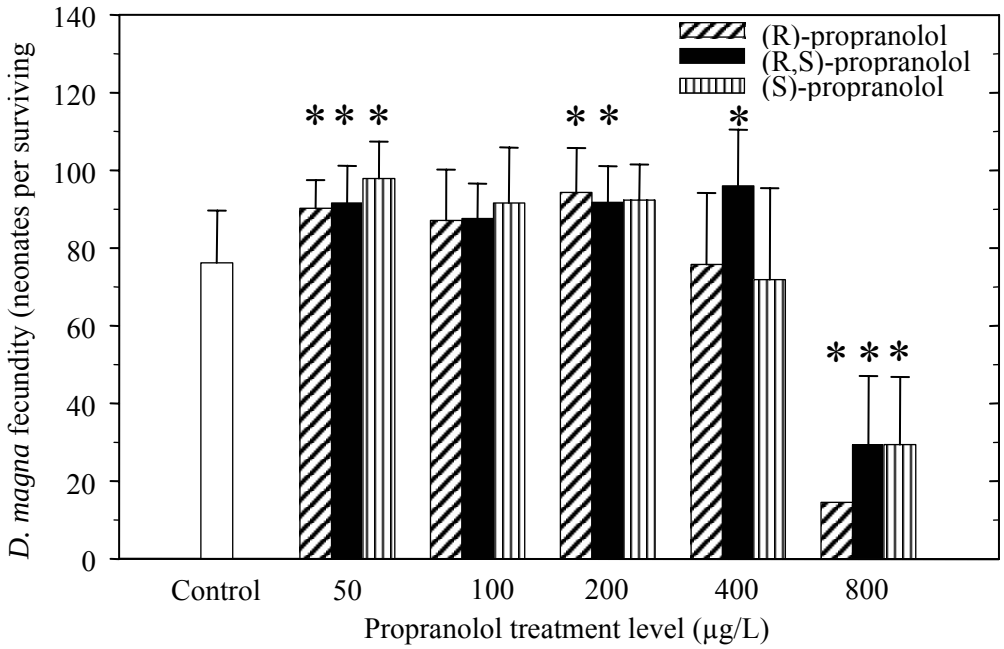

Figure 2.2 *Daphnia magna* reproduction response following a 21-d chronic exposure to *R*-, *rac*-, or *S*-propranolol (n = 10; ANOVA with Bonferroni t-test; error bars are ± 1 standard deviation; asterisks indicate a statistically significant difference from the control group; $\alpha = 0.05$). See text for analytically measured concentrations of propranolol effect levels. Immobilization lowest observed effect concentrations (LOECs) for this exposure were 409.3, 843.7, and >869.0 µg/L for *R*-, *rac*-, and *S*-propranolol, respectively.

2.20); *rac*-propranolol – 1.09 mg/L (0.89, 1.29) and 1.33 mg/L (1.10, 1.56). In the *P. promelas* seven-day short- term chronic test, percent survival was significantly lower than controls for both enantiomers and the racemic mixture at the 750 µg/L treatment level. Results from the assessment of the growth endpoint (as mean weight per surviving organism) showed *S*- and *rac*-propranolol (LOECs = 134.4 and 128.2 µg/L, respectively) to be more toxic than *R*-propranolol (LOEC > 463.6 µg/L) (Figure 2.4).

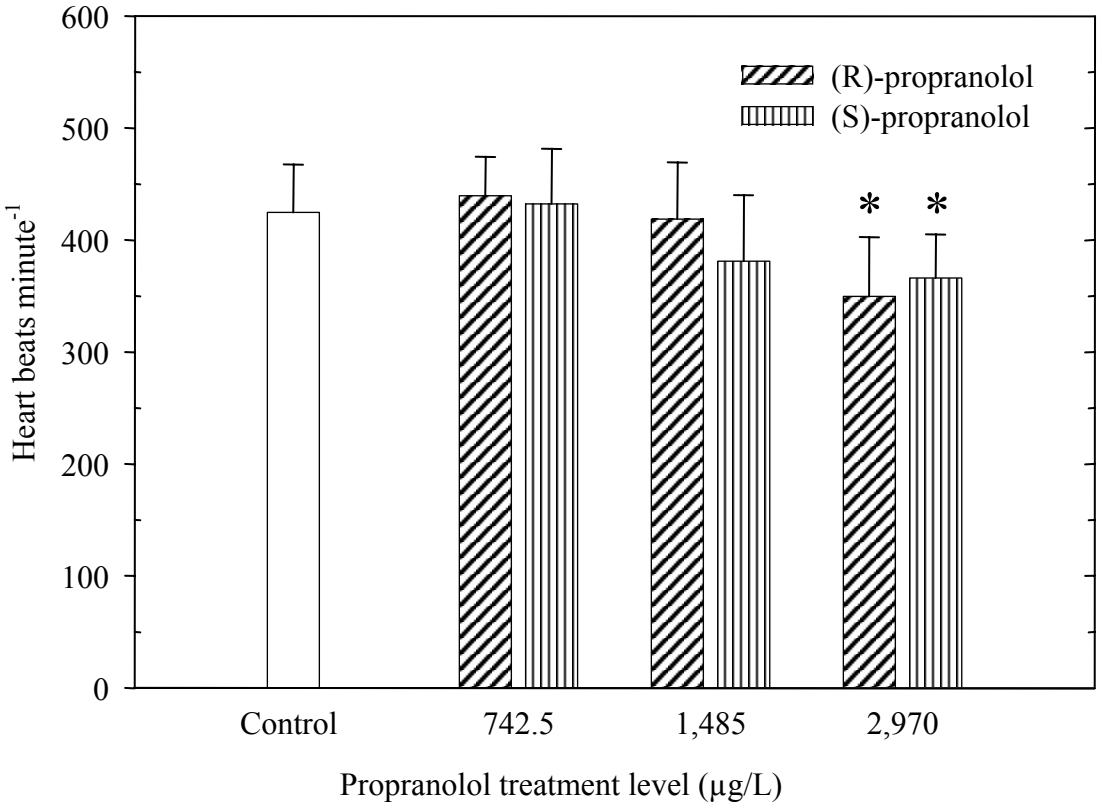

Figure 2.3 Effects on *Daphnia magna* heart rate after 30 minute exposures to *R*- or *S*-propranolol. See text for analytically measured concentrations of propranolol effect levels. (n = 10; ANOVA with Dunnett's test; error bars are ± 1 standard deviation; asterisks indicate a statistically significant difference from the control group; $\alpha = 0.05$)

Discussion

The objectives of this study were to investigate enantiospecific toxicity of propranolol to *D. magna* and *P. promelas* and to examine the effects of acute exposure to propranolol enantiomers on *D. magna* heart rate. Results from this study will aid in developing more accurate assessments of risk for propranolol that consider enantiospecific differences in toxicity. To our knowledge, this research presents the first study of enantiospecific toxicity of a chiral pharmaceutical contaminant to aquatic organisms.

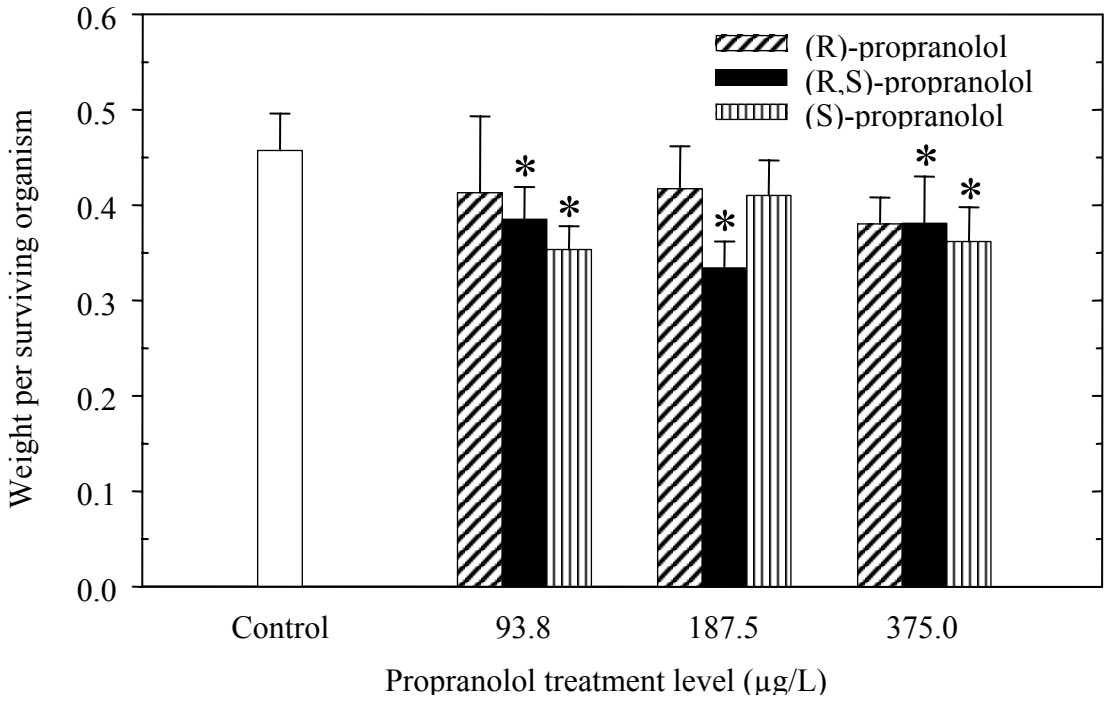

Figure 2.4 *Pimephales promelas* growth response following a 7-d exposure to *R*-, *rac*-, or *S*-propranolol. See text for analytically measured concentrations of propranolol effect levels. (n = 8; ANOVA with Dunnett's test; error bars are ± 1 standard deviation; asterisks indicate a statistically significant difference from the control group; $\alpha = 0.05$)

The average *D. magna* 48-h EC50 for *rac*-propranolol of 1.67 mg/L calculated in this study is roughly equivalent to the average 48-h LC50 value of 1.6 ± 0.3 mg/L propranolol previously reported by Huggett et al. (2002) and in the same range as the 48-h EC50 of 2.75 mg/L propranolol reported by Ferrari et al. (2004) and the 24-h EC50 value of 2.5 mg/L propranolol HCl reported by Lilius et al. (1995). Average 48-h EC50s for each of the three enantiomer treatments in this study were similar, ranging from 1.40 to 1.67 mg/L. This range is much less than the 60-100x difference in enantiospecific activity reported for mammals reported by Howe and Shanks (1966) and Barrett and Cullum (1968). This suggests the acute toxicity of propranolol to *D. magna* observed in

this study is the result of a non-enantioselective process. Therefore, acute toxicity is likely the result of a mechanism other than antagonism of β-receptors, possibly narcosis. Narcosis is non-specific toxicity via disruption of membrane integrity (Cleuvers 2005). Cleuvers (2005) showed that acute propranolol toxicity to *D. magna* is likely the result of narcosis by using structure activity relationships to compare predicted toxicity with empirically measured toxicity data.

Chronic *D. magna* immobilization responses in the 21-d test followed the relationship: *R*-propranolol (most toxic) > *rac*-propranolol > *S*-propranolol (least toxic). Interestingly, increases in *D. magna* reproductive output were observed in all treatments at the 50 µg/L treatment level, for *R*- and *rac*-propranolol at the 200 µg/L treatment level, and at the 400 µg/L treatment level of *rac*-propranolol (Figure 2.2). The mechanistic cause of this increase is unknown at this time and warrants further study. The *D. magna* immobilization LOEC for the *S*-enantiomer (> 869.0 µg/L) was greater than twice that for the *R*-enantiomer (409.3 µg/L), contrary to our hypothesis. Because of this deviation, these results also suggest the mechanism for propranolol toxicity to the immobilization endpoint in chronic *D. magna* exposures is a mechanism other than antagonism of a β-receptor. In fact, the presence of β-receptors has not been reported in *D. magna* or other crustaceans (Dzialowski et al. 2006, Huggett et al. 2002). Postmes et al. (1989) showed that the negative chronotropic effects of the agonist epinephrine could not be blocked by the antagonist propranolol in *D. magna*, suggesting that the drug's actions as well as normal regulation of heart frequency in this species are not mediated through adrenoreceptors.

Recent studies indicate that mammalian pharmacological safety information may be useful to predict fish responses to therapeutics such as propranolol (Huggett et al. 2003b). The results of this study do not support the use of enantiospecific propranolol activity data from vertebrates to predict enantiospecific ecotoxicity test responses in cladocerans, and potentially other invertebrates. However, it is worth noting that some correspondence between the relative toxicities of different β-blockers to *Ceriodaphnia dubia* and mice has been demonstrated, with less correspondence occurring in rats (Fraysse and Garric 2005).

Enantiospecific differences in heart rate reduction were not observed in the acute 30 minute exposures of *D. magna* to propranolol enantiomers, which suggests the absence of a β-receptor mediated effect in short term exposures. The LOECs of 2.61 and 2.62 mg/L for *R*- and *S*-propranolol, respectively, from the acute 30 minute propranolol exposure from the *D. magna* heart rate study was approximately 3.3 times higher than the LOEC of 0.8 mg/L for *rac*-propranolol reported by Dzialowski et al. (2006) in a similar study. Effects of *rac*-propranolol on heart rate were not measured in this study. Dzialowski et al. (2006) showed significant reductions in heart rate to both F0 and F1 generations of *D. magna* in 9-d propranolol exposures at the lowest treatment level tested in that study of 0.055 mg/L. Heart rate was a more sensitive endpoint than immobilization, growth, or reproduction in that study. Further studies of the effects of chronic propranolol exposure on *D. magna* heart rate at lower concentrations and greater durations are warranted to further describe the sensitivity of this endpoint and the mechanism by which propranolol affects the myogenic *D. magna* heart. Because propranolol is also known to be a serotonin antagonist, some have suggested the

possibility that propranolol may elicit an effect through a serotonin-like receptor (Huggett et al. 2002).

The average *P. promelas rac*-propranolol 48-h LC50 of 1.21 mg/L measured in this study is an order of magnitude lower than the Japanese medaka (*Oryzias latipes*) 48-h LC50 of 24.3 mg/L reported by Huggett et al. (2002). Also, in this study a significant decrease in *P. promelas* growth was observed at 128.2 µg/L *rac*-propranolol in a seven-day exposure, where Huggett et al. (2002) reported a significant decrease in growth in medaka at 500 µg/L in a 14-d exposure. Thus, *P. promelas* appear to be more sensitive to propranolol exposure than medaka.

The average 48-h acute LC50s calculated for *P. promelas* were similar for each enantiomer treatment tested, ranging from 1.21 to 1.69 mg/L. Also, no enantiospecific differences in *P. promela*s survival were seen in the seven-day exposure. Therefore, it appears that either acute toxicity is also not mediated by β-receptors in fish, or the survival endpoint is not sensitive enough to demonstrate enantiospecific differences in toxicity at the test durations used. However, *S*- and *rac*-propranolol were more toxic to the growth endpoint in the 7-d exposure than the *R*-enantiomer (Figure 2.4). This was the relative toxicity hypothesized a priori. Because a significant decrease in fish weight was not observed at the highest non-lethal treatment level of *R*-propranolol, 463.6 µg/L, the magnitude of the enantiospecific difference in toxicity to the growth endpoint cannot be compared. However, while non-significant, a decrease in weight occurred at this treatment level (Figure 2.4). Previous studies have shown that teleost species possess β-adrenergic receptors (Laurent et al. 1983, Gamperl et al. 1994). This conservation of receptor type between mammals and fish is a possible explanation for why chronic

enantiospecific fish responses in this study were consistent with those observed in mammal species by Howe and Shanks (1966) and Barrett and Cullum (1968). However, because a racemic mixture is made up of a 1:1 ratio of enantiomers and teleost species are known to possess β-receptors, we hypothesized the toxicity of *rac*-propranolol to be intermediate to that of *R*- and *S*-propranolol in the *P. promelas* chronic study. This was not the case as survival LOECs were equal among *R*-, *S*-, and *rac*-propranolol treatments and growth LOECs were 128.2, 134.4, and > 436.6 µg/L in *rac*-, *S*-, and *R*-propranolol, respectively. It is possible that the hypothesized relative toxicity of *S*-propranolol > *rac*-propranolol > *R*-propranolol would be observed if lower treatment levels of *rac*- and *S*-propranolol were used. It may also be possible that intermediate toxicity was not observed in *rac*-propranolol because of interactions between the two enantiomers. Two enantiomers of a racemic drug may interact with each other producing different pharmacodynamic and pharmacokinetic profiles (Mehvar and Brocks 2001).

In the present study, all significant effects on response variables were seen at concentrations above reported measured environmental concentrations. The lowest treatment causing a significant effect was 50 µg/L of *R*- and *rac*-propranolol causing a significant increase in reproduction in the 21-d *D. magna* exposure. Using a hazard quotient approach suggests that at environmentally measured levels, short-term exposure to propranolol likely represents a low hazard to the endpoints tested in *D. magna* and *P. promelas*. However, Huggett et al. (2002) found that medaka reproduction was impaired at environmentally relevant levels as low as 0.5 µg/L in four week exposures. Because this study has shown survival and growth responses to propranolol to be more sensitive in *P. promelas* than survival and growth endpoints reported by Huggett et al. (2002) for

medaka, additional longer duration assessments of propranolol toxicity to *P. promelas* that include assessments of effects on reproduction are warranted. Also, because short-term laboratory toxicity tests in this study indicated response levels above environmentally measured concentrations does not necessarily preclude deleterious environmental effects. Organisms living in effluent dominated streams, common in arid regions, may experience whole life cycle exposures to a myriad of pharmaceutical and other contaminants (Brooks et al. 2003a), and experimental toxicity studies with durations of longer than a few weeks are rare. Knowledge of aquatic organisms' responses to even simple mixtures of pharmaceuticals is very limited, and additive responses are possible in contaminants with similar modes of action (Kolpin et al. 2002). The fact that a greater than three fold difference in toxicity to *P. promelas* growth between enantiomers was shown to exist in this study illustrates that enantiospecific toxicity of chiral pharmaceutical contaminants should be considered when performing ecological risk assessments of this class of contaminant. Depending on the outcome of experimental investigations into the enantiospecific fate and toxicity of chiral compounds that are distributed as racemates, each enantiomer may need to be considered as a separate compound rather than treating racemic mixtures as one toxicant with a single ecotoxicological profile and identical biotransformation characteristics.

CHAPTER THREE

Enantiospecific Sublethal Effects of the Antidepressant Fluoxetine to a Model Aquatic Vertebrate and Invertebrate

Introduction

In recent years, there has been increasing awareness of the widespread distribution of low concentrations of pharmaceutical compounds in the aquatic environment due to advances in analytical techniques (Daughton and Ternes 1999, Kolpin et al. 2002, Metcalfe et al. 2003). These pharmaceuticals can be human or veterinary therapeutics and are primarily introduced to the environment via treated municipal wastewater or combined sewer overflows (Glassmeyer et al. 2005). Pharmaceuticals can also enter aquatic systems through improper disposal of unused products or by runoff from agricultural operations (Heberer 2002). Continuous release of emerging pharmaceutical contaminants from wastewater treatment plants has resulted in use of the term "pseudopersisent" to describe the ultimate environmental fate of these compounds since the rates of effluent introduction often exceed emerging contaminant half-lives in a receiving system (Brooks et al. 2006).

A percentage of the therapeutic dose of many pharmaceuticals is known to be excreted from humans as the unchanged parent compound. Alternately, conjugated metabolites (e.g., glucoronides) may be cleaved by processes such as microbial degradation and hydrolysis in or after the wastewater treatment process, releasing the parent compound into the aquatic environment (Daughton and Ternes 1999, Heberer 2002). In addition to their presence in ambient samples, recent studies have

demonstrated the ability of some pharmaceutical compounds to bioaccumulate in the tissues of aquatic invertebrates (Capone et al.1996, Brooks et al. 2004, Liebig et al. 2005), fish (Björklund et al. 1990, Brooks et al. 2005, Mimeault et al. 2005), and birds (Oaks et al. 2004). Potential consequences associated with the presence of these biologically active compounds in effluents, surface waters, and animal tissues are not yet fully understood. Sublethal effects are particularly relevant to organisms residing in effluent-dominated streams, which may receive lifetime exposures to emerging contaminants (Brooks et al. 2005, Brooks et al. 2006).

Mammalian pharmacodynamic, pharmacokinetic, and toxicological information generated during drug development may be leveraged to focus ecotoxicological research on compounds with greater potential for sublethal impacts on aquatic ecosystems (Seiler 2002, Huggett et al. 2003b). Aquatic vertebrates (e.g., teleost fish) are believed to have many similar biotransformation enzyme and target receptor systems to humans, with homologies for many important systems ranging from 31-88% (Evans 1993, Huggett et al. 2003b). However, less is known about enzyme and receptor homology of humans and the phylogenetically more distant invertebrates. Thus, an increased understanding of evolutionary relationships of receptor and enzyme homologies among organisms is needed to support ecological risk assessment of emerging contaminants for which sublethal effects may be critically important. Herein, compounds with known vertebrate activities, such as pharmaceuticals, may be used as tools to elucidate mechanisms of action in aquatic species.

Many pharmaceutical compounds and other xenobiotics (e.g., select pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and musk fragrances) are

chiral and distributed as racemic mixtures. Enantiomers have identical molecular formulae and identical physical and chemical properties; however, they can differ markedly in potency, toxicity, bioavailability, and environmental fate due to the stereospecific nature of biological receptors and enzymes (McConathy and Owens 2003, Ali and Aboul-Enein 2004, Stanley et al. 2006). There is a trend in the pharmaceutical industry to develop single enantiomer forms of chiral drugs, which can reduce potential side effects of less potent enantiomers with sometimes poorly understood biological activities. However, Tran et al. (2004) reported that only 12% of chiral synthetic drugs on the market were distributed as single enantiomers, indicating that the majority of chiral pharmaceuticals are still distributed as racemic mixtures.

While enantiospecific differences in fate and effects for chiral contaminants are increasingly recognized, these differences have largely been ignored for environmental pharmaceutical contaminants. Previous research has demonstrated that chiral pharmaceuticals that are distributed as racemic mixtures may not be present in aquatic systems in the 1:1 ratio of the racemate (Fono and Sedlak 2005, Wong 2006). Reasons for such a departure from the racemic mixture include preferential metabolism and/or excretion by the human or animal taking the drug or microorganisms degrading chiral compounds via stereospecific enzymatic processes (Hegeman and Laane 2002). Such a change in enantiomer ratio when there are enantiospecific differences in environmental fates and potencies introduces uncertainty to the exposure and effect analysis components of ecological risk assessments for chiral pharmaceuticals. However, comparison of enantiospecific activity of a chiral drug in mammals with enantiospecific activity/toxicity in aquatic biota may decrease the uncertainty associated with predicting aquatic organism

responses based on mammalian data. Thus, data on the environmental fate and effects of each enantiomer of widely used racemic pharmaceutical mixtures are needed.

To examine enantiospecific sublethal effects on model aquatic organisms, I chose fluoxetine, a widely prescribed selective serotonin reuptake inhibitor (SSRI) that is distributed as a racemic mixture of *R*- and *S*-fluoxetine hydrochloride ($C_{17}H_8F_3NO{\cdot}HCl$, molecular weight = 345.79), as a model compound for this study (Figure 3.1). Fluoxetine

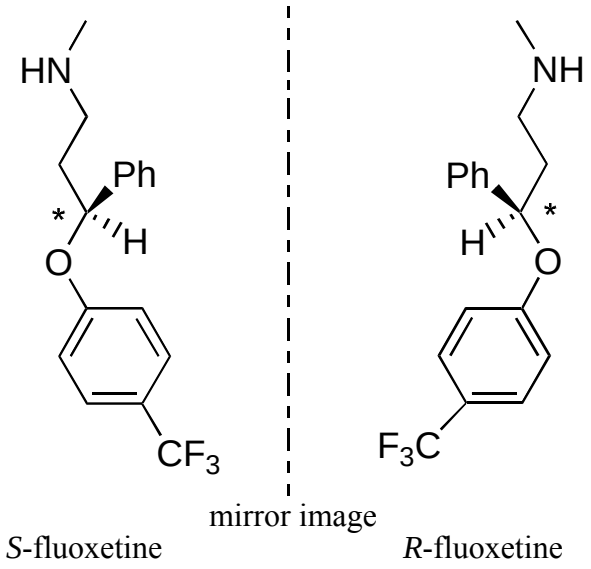

Figure 3.1 Structure and configuration of *S*- and *R*-fluoxetine. Stars indicate chiral carbons.

is indicated for the treatment of depression, obsessive-compulsive disorder, bulimia nervosa, and panic disorder. With approximately 21,403,0000 prescriptions dispensed in 2005, fluoxetine was the 29th most widely prescribed pharmaceutical in the United States that year (www.RxList.com, accessed 10-26-06). Fluoxetine is excreted from the human body primarily via the urine, and approximately 2.0-11.0% of the administered dose is excreted as the unchanged parent compound (Altamura et al. 1994).

Fluoxetine has been measured in surface waters at 0.0055 (USA), 0.012 (USA), and 0.013-0.046 µg/L (Canada) by Vanderford et al. (2003), Kolpin et al. (2002), and Metcalfe et al. (2003), respectively. Higher concentrations of 0.038–0.099 µg/L (Canada) (Metcalfe et al. 2003) and 0.54 µg/L (USA) (Weston et al. 2001) have been measured in municipal effluents. Fluoxetine has also been measured in biosolids and sediments at mean concentrations of 37.4 and 1.84 µg/kg, respectively (USA) (Furlong et al. 2004). Brooks et al. (2005) detected fluoxetine and norfluoxetine in brain, liver, and muscle tissues of three different fish species living in an effluent dominated stream in north Texas, USA. The highest concentrations of fluoxetine and norfluoxetine were measured in fish brain tissue at 1.58 ± 0.74 ng/g and 8.86 ± 5.9 ng/g, respectively.

The occurrence of fluoxetine in surface waters and in aquatic organisms has raised concern because serotonin modulates a wide range of physiological processes in aquatic vertebrates and invertebrates (reviewed in Fong (2001)). For example, previous studies of teleost fish have demonstrated that immune response (Ferriere et al. 1996), reproduction (Khan and Thomas 1992), thermal acclimation (Tsai and Wang 1997), swimming activity (Fingerman 1976), feeding (de Pedro et al. 1998), and social aggression (Adams et al. 1996, Perreault et al. 2003) are modulated, at least in part, by serotonin. In invertebrates, serotonin has been shown to play a role in the physiology and behavior of many different taxa. For example, aggressive behavior in crustaceans (Huber et al. 1997), induction of spawning in bivalves (Ram et al. 1993), cilia-driven rotational movement in gastropod embryos (Uhler et al. 2000), and swimming in annelids (Brodfuehrer et al. 1995) have all been shown to be influenced by serotonergic control.

Both fluoxetine enantiomers are approximately equipotent serotonin reuptake inhibitors (Wong et al. 1988, Baumann et al. 2002). However, the demethylated metabolite of *R*-fluoxetine, *R*-norfluoxetine, has been shown to be approximately 20-fold less potent than *S*-norfluoxetine in rat studies (Wong et al. 1993), making fluoxetine an attractive model compound to study sublethal responses to enantiomers with differing biological activity. Our primary objective was to assess potential enantiospecific differences with standardized and behavioral sublethal responses of the model organisms *Pimephales promelas* (fathead minnow, vertebrate) and *Daphnia magna* (water flea, invertebrate) to this widely-used chiral pharmaceutical. Previous research in our laboratory identified that potency differences for different enantiomers may provide some insight to enantiospecific toxicity (Stanley et al. 2006). Thus, our secondary objective was to test the utility of employing a chiral pharmaceutical as a model compound for studying enantiospecific toxicity of chiral contaminants. Our hypothesis was that *S*-fluoxetine would be more toxic to sublethal standardized and behavioral endpoints in *D. magna* and *P. promelas* because of the known greater potency of the primary metabolite *S*-norfluoxetine. The endpoints of *P. promelas* feeding rate and *D. magna* grazing rate were assessed in addition to the traditional standardized toxicity testing endpoints of immobilization, survival, growth, and reproduction to support an assessment of the potential ecological consequences of fluoxetine exposure. In addition, because pharmaceuticals are generally present in the environment at trace (ng/L) concentrations, traditional ecotoxicity endpoints may not be sufficiently sensitive to adequately characterize aquatic risk associated with fluoxetine exposure (Brooks et al. 2003a). Changes in behaviors such as feeding may be the initial response of an organism to a

chemical stressor and may also explain observed reductions in survival, growth, or reproduction (Fernández-Casalderry et al. 1994).

Materials and Methods

Control and dilution water for all experiments was reconstituted hard water (RHW) made according to U.S. Environmental Protection Agency (U.S. EPA) methods (U.S. EPA 1992). All exposures were carried out in a controlled environmental chamber. Temperature in this chamber was held constant at 25 ± 1 °C, and the exposure chamber was kept on a 16:8 hour light-dark ratio for the duration of all exposures. Specific conductance (µs/cm), pH, and dissolved oxygen (mg/L) of all test solutions were measured before acute and chronic test initiation and at renewal of chronic tests using a multiprobe according to standard methods (APHA, AWWA, WEF 1998). Alkalinity (mg/L as $CaCO_3$) and hardness (mg/L as $CaCO_3$) were also measured amperiometrically and by colorimetric titration, respectively, according to standard methods (APHA, AWWA, WEF 1998). Mean (± one standard deviation) RHW water quality parameters for all experiments were as follows: pH = 8.4 (± 0.3), dissolved oxygen = 7.3 mg/L (± 1.4), specific conductance = 557.3 µs/ cm (± 19.8), hardness = 171.0 mg/L as $CaCO_3$ (± 8.7), and alkalinity = 112.5 mg/L as $CaCO_3$ (± 11.6). Each parameter fell within test acceptability ranges (U.S. EPA 2002a, U.S. EPA 2002b, OECD 1998). Fluoxetine was obtained as *R*-, *rac*- (racemic), and *S*-fluoxetine hydrochloride from Sigma-Aldrich (St. Louis, MO, USA). All fluoxetine concentrations are reported as fluoxetine and not fluoxetine hydrochloride. A solvent carrier was not required to dissolve fluoxetine hydrochloride into the RHW.

P. promelas *Testing*

Two *P. promelas* 48-h acute studies were performed according to U.S. EPA Method 2000.0 (U.S. EPA 2002a). These exposures were carried out in 600 ml glass beakers with 10 organisms per beaker and four replicates per treatment level. The volume of media per beaker was 250 ml. Treatment levels were set using a 0.5 dilution series and ranged from 50 to 1,600 µg/L fluoxetine. Four-day-old fish were used to initiate the first acute exposures, while six-day-old fish were employed for the second study. Juvenile fish were fed newly hatched brine shrimp (*Artemia* sp.) nauplii two hours before test initiation and were not fed during the 48-h exposure period. Survival was the lone endpoint measured in these acute exposures.

Effects of aqueous exposure to fluoxetine enantiomers on *P. promelas* survival and growth were assessed in a seven-day short-term chronic study that followed a modified version of U.S. EPA Method 1000.0 (U.S. EPA 2002b). *P. promelas* were exposed in 600 ml glass beakers with a test volume of 250 ml. This was a static renewal test, renewed daily. Nominal treatment levels of *R*-, *rac*-, and *S*-fluoxetine for this experiment were 1, 10, 50, 100, and 250 µg/L. Juvenile *P. promelas* were fed newly hatched brine shrimp (*Artemia* sp.) nauplii twice a day according to U.S. EPA methods (U.S. EPA 2002b). In addition to the survival and growth endpoints regularly assessed in this method, enantiospecific effects of exposure to *R*-, *rac*-, and *S*-fluoxetine on *P. promelas* feeding rate were assessed after completion of the 7-day exposure period. Effects on feeding rate were determined by enumerating the number of brine shrimp nauplii consumed in a 15-minute time period. Food was withheld from the fish for 24-hours prior to the feeding rate study. Two fish from each replicate beaker (eight fish per

treatment level) were selected at random to be used in the feeding study. Each of these fish was individually placed into a 100 ml glass beaker containing 100 ml of RHW one hour before addition of 25 brine shrimp nauplii. Prior to addition to test beakers, the brine shrimp were first transferred to a beaker containing RHW in order to avoid adding excess salts to the test containers. Following a 15-minute feeding period, the fish was removed, and the remaining brine shrimp were preserved in Lugol's solution for later enumeration using a stereomicroscope. Feeding rate was recorded as the number of brine shrimp nauplii consumed per minute. The number of *Artemia* consumed by the two fish representing each experimental unit was averaged to calculate the feeding rate for an experimental unit. Fish that were not used for feeding trials were used to analyze growth responses, measured as dry weight.

D. magna *Testing*

A 21-day *D. magna* chronic toxicity test was performed following a procedure adapted from the Organisation for Economic Co-operation and Development (OECD) (1998) and the U.S. EPA (1996). Endpoints assessed were *D. magna* immobilization, reproduction (young per organism), and grazing rate. Less than 24-h-old *D. magna* were used to initiate this test. Nominal treatment levels for this experiment were 10, 50, 100, 250, 500, and 1000 µg/L of *R*-, *rac*-, and *S*-fluoxetine. Test containers were 30 ml disposable plastic cups with a test volume of 30 ml. This was a static renewal test. Test media was renewed every other day. *D. magna* were fed 0.6 ml/day of a mixture of *P. subcapitata* and cereal grass media (U.S. EPA 2002a, Hemming et al. 2002). At the end of the 21-day exposure period, food was withheld from the *D. magna* for 24-hours. Three organisms from each treatment level that was not significantly different than

controls for immobilization were randomly selected for assessment of fluoxetine exposure on grazing rate. Only organisms that, upon examination, did not have young in their brood pouch were considered for use in order to prevent the release of neonates during the assessment of grazing rate. Organisms were individually placed into a 30 ml plastic cup containing an initial *P. subcapitata* cell density of approximately 4.1 x 10^6 cells/ml in RHW and allowed to feed for 18 hours. A standard curve relationship was initially established for green algae cell number and fluorescence. Subsequently, absorbance at 750 nm measured by fluorometer was used as a surrogate for algal cell counts according to U.S. EPA Method 1003.0 (U.S. EPA 2002b). This initial cell density was chosen to approximate the algal cell density of normal culture and toxicity test feedings. An initial fluorescence reading was taken in each test container prior to the addition of organisms. Following the 18-h exposure period, the *D. magna* were removed and a final fluorescence reading was taken. Grazing rate was recorded as the number of cells consumed per hour.

Fluoxetine Quantitation

Aqueous fluoxetine concentrations from all toxicity tests were quantitated using a benchtop liquid chromatograph-tandem mass spectrometer (LC-MS/MS) consisting of a Varian ProStar® HPLC system coupled to a Varian Model 1200L triple-quadrupole mass analyzer (Varian, Inc., Palo Alto, CA, USA). Bioaccumulation of fluoxetine was not measured in this study. Because all toxicity tests were performed with either single fluoxetine enantiomers or the racemic mixture, chiral separation of fluoxetine enantiomers was not required in this study. Chromatographic separation of fluoxetine and 10,11-dihydrocarbamazepine (internal standard) was achieved using a narrow-bore

15 cm × 2.1 mm id (5 μm, 80 Å) Zorbax extend-C18 column (Agilent technologies, USA) with isocratic elution at a flow rate of 0.30 ml/minute. The mobile phase consisted of mixture of 55% 0.1% (v/v) aqueous formic acid and 45% methanol. An autoinjector, in partial loop mode, was used to achieve a reproducible injection volume of 10 μL for each run. The mass spectrometer was operated using positive electrospray ionization. Detector, needle, and shield voltages were set to 1.4 kV, 5.0 kV, and 0.6 kV, respectively. The MS/MS transitions monitored for detection and quantitation purposes were *m*/*z* 310>148 for fluoxetine and *m/z* 239>194 for internal standard at collision energies of 5.5 V and 20.0 V, respectively.

Analyte concentrations in both control and toxicological samples were determined using an internal standard calibration procedure. The response factor was calculated by dividing the peak area for fluoxetine by the peak area for the internal standard, and a calibration curve was prepared by plotting a linear regression ($r^2 \geq 0.998$) of the response factor versus analyte concentration for all calibrators analyzed. Instrument calibration was monitored through the use of continuing calibration verification (CCV) samples with an acceptability criterion of ± 20%. In a given run, one blank and one CCV sample were interspersed between every five toxicological samples for quality assurance purposes. Reported analytical concentrations of fluoxetine in toxicological samples represent the mean concentration for triplicate sample injections plus or minus one standard deviation. On average, across all toxicity tests, measured concentrations were 89.7% ± 12.5% as compared to nominal treatment levels.

Statistical Analyses

Statistical significance of response variables was assigned at $\alpha = 0.05$ for all tests. The 50% lethal concentration (LC50) values for the 48-h *P. promelas* acute tests were calculated using the probit method if possible; however, if assumptions of the probit method were not met, the Trimmed Spearman-Karber method was used. Proportional mortality data were arc sine (square root (y)) transformed prior to hypothesis testing. A Fisher's Exact Test was used for the immobilization endpoint in the *D. magna* 21-d chronic test. A Steel's Many-One Rank test was used to analyze *P. promelas* survival in the 7-d chronic test. Analyses of the *P. promelas* feeding rate and growth endpoints as well as the *D. magna* grazing rate endpoint was performed using parametric one-way analysis of variance (ANOVA) along with a Dunnett's multiple range test. The *D. magna* reproduction endpoint was assessed using a Steel's Many-One Rank Test or a Wilcoxon Rank Sum Test with Bonferroni adjustment, as appropriate, according to (U.S. EPA 2002b).

Results

P. promelas *Testing Results*

Average 48-h LC50s for the two *P. promelas* acute studies performed were 212, 198, and 216 µg/L for *R*-, *rac*-, and *S*-fluoxetine, respectively. Results from the assessment of the growth and survival endpoints in the 7-day *P. promelas* exposure can be found in Figure 3.2. *S*-fluoxetine was the most toxic form to the survival endpoint with a lowest observed effect concentration (LOEC) of 101 µg/L. The survival LOECs for *R*- and *rac*-fluoxetine were 170 and 174 µg/L, respectively. Growth was significantly reduced at the 53 and 51 µg/L treatment levels for *rac*- and *S*-fluoxetine, respectively. *R*-

fluoxetine did not significantly affect growth at concentrations that were not statistically different for survival.

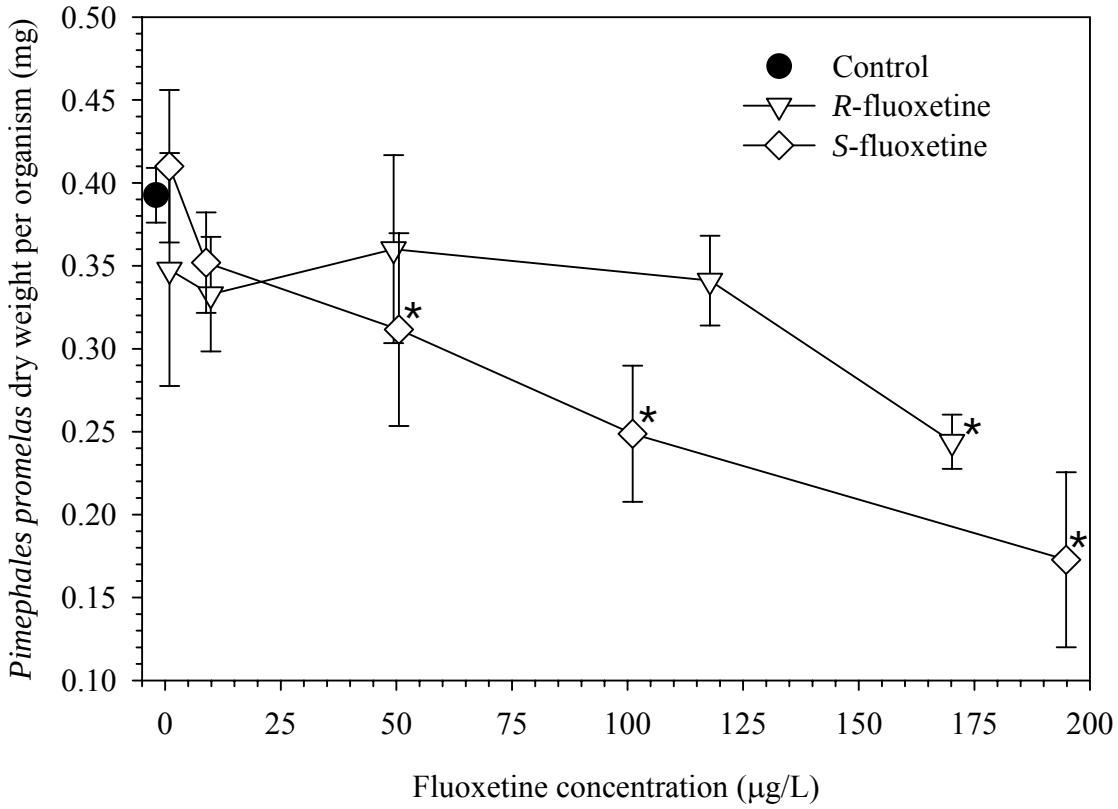

Figure 3.2 Effects on *Pimephales promelas* growth after seven-day exposure to *R*-, and *S*-fluoxetine ($n = 4$; ANOVA with Dunnett's test; error bars are ± 1 standard deviation; stars indicate a statistically significant difference from the control group; $\alpha = 0.05$).

Results from the assessment of the *P. promelas* feeding rate endpoint can be found in Figure 3.3. Feeding rate was reduced in a dose-dependent manner for all fluoxetine treatments. *P. promelas* feeding rate was most sensitive to *S*-fluoxetine

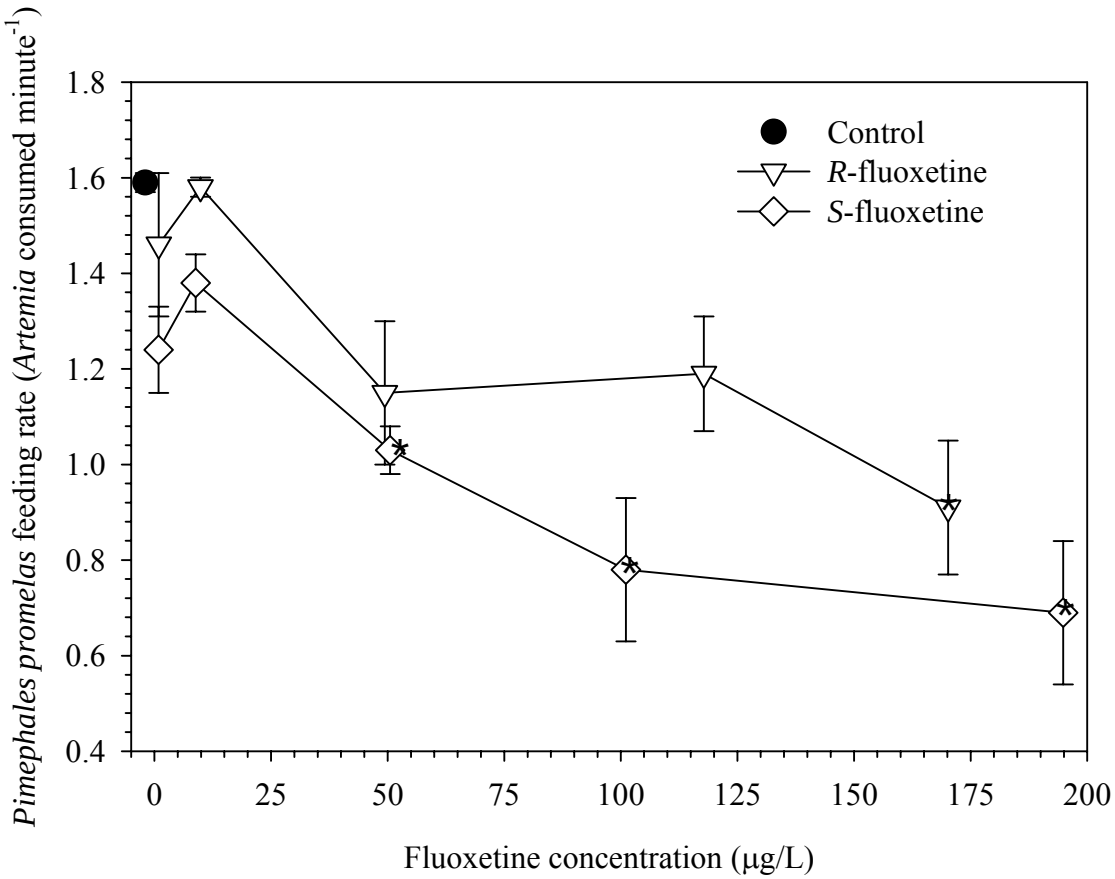

Figure 3.3 *Pimephales promelas* feeding rate response to seven-day exposure to *R*- and *S*-fluoxetine ($n = 4$; ANOVA with Dunnett's test; error bars are ± 1 standard error; stars indicate a statistically significant difference from the control group; $\alpha = 0.05$).

with a LOEC of 51 µg/L. LOECs of 170 and 106 µg/L were observed for *R*- and *rac*-fluoxetine, respectively.

D. magna *Testing Results*

The immobilization LOECs for the 21-day chronic *D. magna* exposure for *R*-, *rac*-, and *S*-fluoxetine were all similar at 429, 430, and 444 µg/L, respectively. There were no significant positive or negative differences in reproduction at treatment levels that were not significantly different for immobilization. Thus, no enantiospecific

differences were observed for immobilization or reproduction. Results from the grazing rate study are presented in Figure 3.4. Interestingly, *D. magna* grazing rate generally

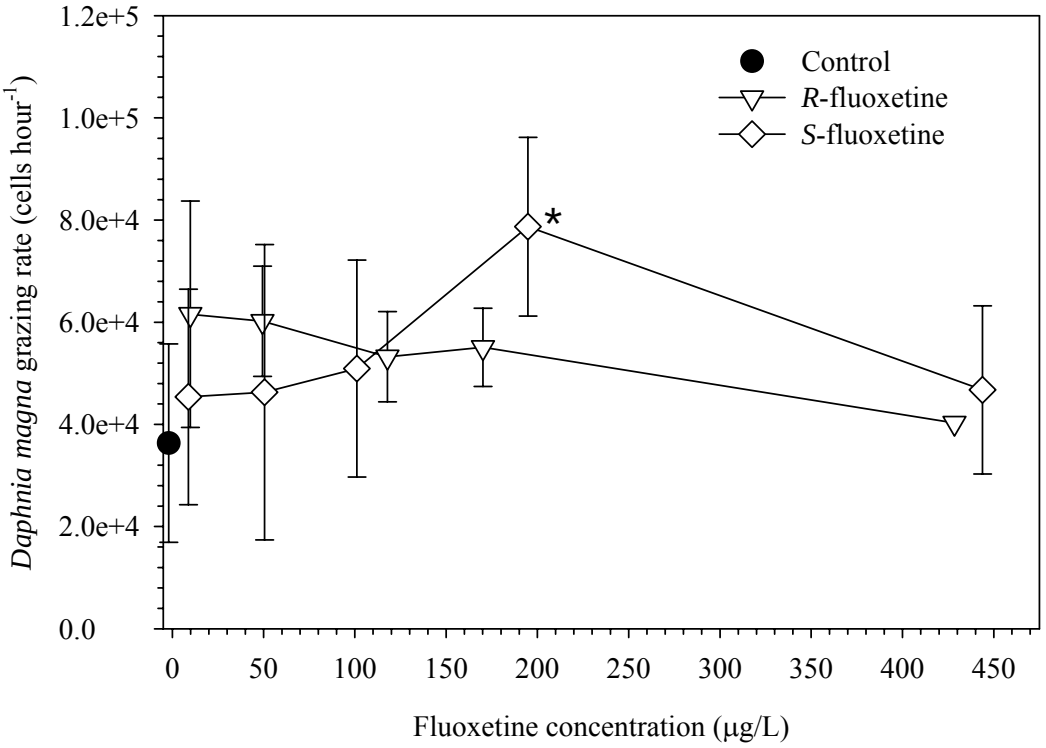

Figure 3.4 Effects on *Daphnia magna* grazing rate after 21-day exposure to *R*- and *S*-fluoxetine (n = 3; ANOVA with Dunnett's test; error bars are ± 1 standard deviation; stars indicate a statistically significant difference from the control group; $\alpha = 0.05$).

increased with fluoxetine enantiomer treatments; however, only the *S*-fluoxetine 195 µg/L treatment was statistically significantly higher than controls. A summary of all chronic ecotoxicological endpoints measured is found in Table 3.1.

Discussion

The objectives of this research included an assessment of potential enantiospecific differences in sublethal effects of the model chiral pharmaceutical fluoxetine by establishing effect data for enantiomers and a comparison of our findings with

mammalian pharmacology data on the enantiospecific differences in fluoxetine enantiomer potency. To our knowledge, this research represents the first study to assess the enantiospecific effects of any chiral contaminant on behavioral and other sublethal endpoints in model aquatic organisms.

The 48-h acute *P. promelas* exposures yielded similar average LC50s for *R*-, *rac*-, and *S*-fluoxetine, ranging only from 198 to 216 µg/L. Thus, no enantiospecific pattern of acute toxicity can readily be identified from these results. Because significant enantiospecific differences in toxicity were not observed, it is possible that acute toxicity was a result of narcosis, a non-specific mode of toxicity attributed to a disruption in membrane integrity. Alternately, the survival endpoint may not be sensitive enough to demonstrate enantiospecific differences in toxicity at the test durations used.

Enantiospecific effects that support our hypothesis of *S*-fluoxetine being more toxic than *R*-fluoxetine were observed in the *P. promelas* seven-day exposure for the survival, growth, and reproduction endpoints. The LOECs for the survival and growth endpoints in this exposure for *R*-fluoxetine are 1.6 and 3.3-fold greater than that for *S*-fluoxetine, respectively (Figure 3.2). The *P. promelas* feeding rate LOEC for *R*-fluoxetine of 170 µg/L is 3.3-fold greater than that of the LOEC for *S*-fluoxetine (Table 3.1). This enantiospecific difference in the effects of fluoxetine on *P. promelas* feeding rate is consistent with the results from mammalian testing reported by Wong et al. (1988) who showed *S*-fluoxetine to be a slightly more potent suppressor of food intake by rats

Table 3.1 Effect and no-effect endpoints (µg/L) from 7-day *Pimephales promelas* and 21-day *Daphnia magna* chronic exposures to R-, *rac*-, and *S*-fluoxetine

	Pimephales promelas			*Daphnia magna*		
	Survival	Growth	Feeding Rate	Immobilization	Reproduction	Grazing Rate
R-fluoxetine						
NOEC	118	118	118	170	170	none
LOEC	170	170	170	429	429	none
rac-fluoxetine						
NOEC	106	9	50	174	174	none
LOEC	174	53	106	430	430	none
S-fluoxetine						
NOEC	51	9	9	195	195	101
LOEC	101	51	51	444	444	195*

* = significant increase relative to controls
NOEC = No observed effect concentration
LOEC = Lowest observed effect concentration

than *R*-fluoxetine. Reductions in behaviors such as feeding rate reveal clear connections between the biochemical, individual, and population levels of biological organization (Weis et al. 2001). Altered predation rates can affect population dynamics of both predator and prey species (Weis et al. 2001).

Increased levels of serotonin in the brain have been shown to be a factor in the inhibition of feeding behavior in fish (de Pedro et al. 1998) and mammals (Blundell and Halford 1998). However, in addition to inducing hypophagia by raising brain serotonin concentrations, fluoxetine is also thought to induce hypophagia via reduction in neuropeptide Y (NPY) concentrations (Dryden et al. 1996). NPY is a powerful central appetite stimulant, causing carbohydrate- and fat-selective hyperphagia in humans (Dryden et al. 1996). NPY is one of the most abundant brain neuropeptides in mammals and is highly concentrated in the hypothalamus (Dryden et al. 1996). NPY has been shown to be widely distributed in the central nervous system of various fish species (Aldegunde and Manceboe 2006). Also, Halford and Blundell (1996) suggest that at high doses in mammals, fluoxetine induced hypophagia may be a result of a general decrease in behavior or sedation response that is mediated by an unspecified catecholaminergic mechanism.

Endpoint sensitivity for the *P. promelas* 7-day test with *rac*-fluoxetine was growth = feeding rate > survival. It is clear that the racemic mixture is not always intermediate in toxicity to the *R*- and *S*-enantiomers (Figures 3.2 and 3.3, Table 3.1), as might be expected. This may be due to variability in measured endpoints or possibly due to an interaction between the two enantiomers themselves. Such interactions are known to occur for enantiomers of some classes of pharmaceuticals (Mehvar and Brocks 2001),

and non-intermediate racemate toxicity of chiral pharmaceuticals to model aquatic organisms has previously been reported by our group (Stanley et al. 2006).

Enantiospecific effects were not observed in the immobilization or reproduction endpoints of the standardized 21-day *D. magna* study. The variable nature of the *D. magna* grazing rate response did not support generalizations about enantiospecific differences in this endpoint. While the mechanism for the observed increase in grazing rate with fluoxetine treatment as compared to controls (Figure 3.4) is unknown at this time, stimulation of other endpoints in cladocera by fluoxetine has previously been observed. Brooks et al. (2003b) observed a significant increase in *Ceriodaphnia dubia* fecundity at one treatment level of fluoxetine (56 µg/L) over a 7-day exposure period, and Flaherty and Dodson (2005) report an increase in *D. magna* reproduction with 36 µg/L fluoxetine treatment in a 30-day chronic study. Although minor (approximately three neonates) increases in the mean number of neonates produced per organism were observed for two treatment levels in this experiment, R-fluoxetine 10 µg/L and *S*-fluoxetine 51 µg/L, these increases were not statistically significant and are likely not ecologically relevant. This study supports the suggestion of Flaherty and Dodson (2005) that *D. magna* exposed to fluoxetine may require a higher minimum food level to survive.

All significant treatment effects in these experiments were observed well above environmentally measured concentrations of fluoxetine (Table 3.1). The lowest treatment level causing an effect was 51 µg/L *S*-fluoxetine causing reductions in fish growth and feeding rate in the 7-day exposure. However, it should be noted that this exposure time is much less than the duration of exposure that aquatic organisms living in effluent dominated systems experience. Effluent-dominated systems are common in arid regions

of the world. Brooks et al. (2006) state that in the United States approximately 23% of regulated effluent releases enter streams receiving less than 10-fold instream dilution, and that during low flow conditions, this percentage increases to approximately 60%. In the south central United States, the majority of permitted dischargers appear to enter effluent-dominated streams (Brooks et al. 2006). *P. promelas* that are more mature than the juveniles employed for this study may be more sensitive to fluoxetine enantiomer exposure than the effect levels reported here. Brooks et al. (2003b) observed an age dependant increase in fluoxetine toxicity, potentially attributed to a development increase in cytochrome P450 activation of fluoxetine to its more active metabolite, norfluoxetine. Also, even though fluoxetine has been shown to bioaccumulate in various fish species (Brooks et al. 2005), the full long-term consequences of this remain unknown. Further, organisms living in effluent dominated systems are generally not exposed to one toxicant at a time. Rather, these organisms are more likely to experience almost continuous exposure to low levels of multiple SSRIs and other contaminants simultaneously. Before toxicity of complex mixtures can be fully understood, the sublethal toxicity profiles of individual contaminants must be known. As evidenced in the current study, behavioral endpoints may provide additional lines of evidence for pharmaceutical effects on aquatic organisms if specific behavioral responses (e.g., feeding, aggression) are likely based on mammalian pharmacological data. However, future studies are required to evaluate target tissue (e.g., 5-HT, 5-HIAA levels) and behavioral responses to specific plasma levels of fluoxetine.

Up to a 13.1-fold difference in no observable effect concentrations (NOECs) and a 3.3-fold difference in LOECs were observed between *R*- and *S*-fluoxetine for sublethal

toxicity to standardized and behavioral endpoints in *P. promelas*. A similar magnitude of difference in toxicity to standardized endpoints between enantiomers was observed by our group for propranolol (β-blocker), another widely used chiral pharmaceutical contaminant that is distributed as a racemic mixture (Stanley et al. 2006). The observation of enantiospecific toxicity for standardized fish endpoints more closely following the hypothesized relationship to mammalian potency than standardized invertebrate responses was also observed with propranolol (Stanley et al. 2006). Thus, enantiospecific mammalian responses are more predictive of sublethal standardized and behavioral aquatic vertebrate responses to fluoxetine than invertebrate responses. Detection of significant enantiospecific differences in sublethal responses suggests that enantiospecific toxicity differences should receive future consideration, and select behavioral endpoints may support weight-of-evidence approaches in ecological risk assessments of chiral emerging contaminants.

CHAPTER FOUR

Chronic sublethal effects of *S*-fluoxetine to adult *Pimephales promelas*

Introduction

Fluoxetine is a selective serotonin reuptake inhibitor pharmaceutical that is distributed as a racemic mixture of *R*- and *S*-fluoxetine hydrochloride ($C_{17}H_8F_3NO \cdot HCl$, molecular weight = 345.79). Fluoxetine has been detected in effluents (Metcalfe et al. 2003), surface waters (Kolpin et al. 2002, Metcalfe et al. 2003, Vanderford et al. 2003), biosolids and sediments (Furlong et al. 2004), and even in brain, liver, and muscle tissues of freshwater fish collected from effluent dominated streams (Brooks et al. 2005). The presence of fluoxetine in the environment has raised concern because serotonin is known to play a role in many physiological processes in aquatic vertebrates and invertebrates (Fong 2001).

Previous research by our group has shown *S*-fluoxetine to be more toxic than *R*-fluoxetine to select endpoints in juvenile *Pimephales promelas* (fathead minnows) (Stanley et al. Accepted with revision). A 13.1-fold difference in no observable effect concentrations (NOECs) and a 3.3-fold difference in lowest observable effect concentrations (LOECs) were observed between fluoxetine enantiomers for the endpoints of growth and feeding rate (Stanley et al. Accepted with revision). These enantiospecific differences in toxicity are consistent with previous studies that have shown the primary metabolite of *S*-fluoxetine, *S*-norfluoxetine, to be up to 20-fold more potent than its antipode in mammalian studies (Wong et al. 1993). *S*- and *R*-fluoxetine are

approximately equally potent serotonin reuptake inhibitors (Wong et al. 1988, Baumann et al. 2002).

Because of the enantiospecific differences in fluoxetine toxicity observed in juvenile *P. promelas*, the goal of the present study was to assess the effects of longer-term (21-d) exposures of adult *P. promelas* to the more toxic enantiomer of fluoxetine, *S*-fluoxetine. I am unaware of any published studies on the effects of fluoxetine treatment on adult fathead minnows, despite the fact that Brooks et al. (2003) observed an age dependant increase in fluoxetine toxicity. This increased toxicity with age in juvenile fathead minnows was theoretically attributed to a developmental increase in cytochrome P450 biotransformation of fluoxetine to norfluoxetine. The present study allows for comparisons of toxicity endpoints between larval and adult fathead minnows.

Huggett et al. (2003b) proposed a model to predict toxicity of aquatic pharmaceutical contaminants to fish by estimating their concentration in fish plasma using their octanol-water partitioning coefficient, Log K_{ow}, to estimate their hydrophobicity and comparing these estimated concentrations to human therapeutic plasma concentrations. I tested this model by measuring plasma fluoxetine concentrations in *P. promelas* and linking these to the bioassay endpoints assessed in the present study. These included survival, fecundity, fertilization rate, hatching success, feeding rate, swimming performance (critical swimming speed using a swim tunnel), and swimming behavior (various endpoints assessed using digital imaging software). Swimming behaviors were assessed because in addition to the effects of SSRI exposure and altered serotonin levels on feeding rate previously discussed elsewhere (Stanley et al. Accepted with revision), SSRI treatment has also been shown to affect other behaviors in

teleosts. Perreault et al. (2003) treated bluehead wrasse, *Thalassoma bifasciatum*, a coral reef fish, with intraperitoneal injections of fluoxetine for two weeks and noted decreased swimming activity and territorial aggression in males with fluoxetine treatment. They also saw similar results in a field study where exposure consisted of a single injection of fluoxetine. Winberg et al. (1993) observed significantly reduced spontaneous locomotor activity in the teleost Arctic charr, *Salvelinus alpinus*, upon treatment via intraperitoneal injection with the SSRI zimeldine.

Materials and Methods

A short-term reproductive *P. promelas* test was conducted based on the experimental design given by Ankley et al. (2001). The present study consisted of a 14-d pre-exposure period to assure reproductive health of the test organisms followed by a 22-d exposure period. The fish used were reproductively mature *P. promelas*, approximately seven months old, with no prior spawning experience.

Fish were exposed in 18 L aquaria with a water volume of 10 L. There were four replicate tanks per treatment level. Each tank contained two male and four female *P. promelas*. Three sections of halved polyvinyl chloride (PVC) pipe were placed at the bottom of each tank as substrates for the attachment of eggs. The exposure and all behavioral assays were carried out in a controlled environmental chamber on a 16:8 hour light-dark ratio. Source water for this test was dechlorinated tap water, dechlorinated via carbon filtration. Each exposure tank was aerated to ensure adequate dissolved oxygen concentration. Fish were fed adult *Artemia*, brine shrimp, ad libitum twice a day throughout the pre-exposure and 21-d exposure periods. Dissolved oxygen and temperature were measured in each tank daily. Hardness, alkalinity, specific

conductance, and total chlorine were checked in a randomly selected tank in each treatment level twice a week. Hardness and alkalinity were measured by colorimetric titration and amperiometrically, respectively, according to standard methods (APHA, AWWA, WEF, 1998). Dissolved oxygen, temperature, and specific conductance were measured using a multiprobe. Total chlorine was measured using a Hach (Loveland, CO, USA) pocket colorimeter.

After the initial 14-d pre-exposure period, fish were exposed to nominal concentrations of *S*-fluoxetine of 0.3, 3, 10, 20, and 50 µg/L plus a control. *S*-fluoxetine was purchased from Sigma (St. Louis, MO, USA). Treatment levels were maintained in exposure tanks using a concentrated stock solution of *S*-fluoxetine and a Mount-Brungs style proportional diluter system (Mount and Brungs 1967) to perform one complete water change in exposure tanks every 24-h period.

Treatment levels were chosen using juvenile *P. promelas* fluoxetine toxicity data (Stanley et al. Accepted with revision) and by using a modified version of the model proposed by Huggett et al. (2003b) to estimate aqueous concentrations of fluoxetine necessary to achieve fish steady state plasma concentrations ($F_{SS}PC$) that are in the range of human therapeutic concentrations, approximately 100-500 µg/L (Table 4.1) (Baldessarini 2001). Specifically, Huggett et al.'s (2003) model includes the following equations. The first was initially described by Fitzsimmons et al. (2001) and is used to describe partitioning between the aqueous phase and fish blood (Log $P_{Blood:Water}$):

$$\text{Log } P_{Blood:Water} = 0.73 \text{ x Log } K_{ow} - 0.88$$

This model assumes the major factor that controls uptake of a dissolved compound into the blood stream of a fish is its hydrophobicity (i.e., Log K_{ow}) and uses this value along

with measured or predicted environmental concentration of a pharmaceutical compound to predict $F_{SS}PC$ (Huggett et al. 2003b). Because fluoxetine is a weak base and is ionizable at environmentally realistic pH levels, I used Advanced Chemistry Development's (Toronto, Ontario, Canada) ACD/I-Lab web service (ACD/LogD 8.02) to predict values of the octanol-water distribution coefficient, Log D, for fluoxetine at our experimental pH (experimental pH = 7.96, calculated Log D = 2.1). I modified the Fitzsimmons et al. (2001) equation given above by using the environmental pH-specific Log D value instead of Log K_{ow}:

$$\text{Log } P_{Blood:Water} = 0.73 \text{ x Log D } -0.88$$

The equation used to estimate fish steady state plasma concentration for a given environmental concentration of contaminant (EC) is:

$$F_{ss}PC = (EC)\ (P_{blood:water})$$

Table 4.1 Nominal *S*-fluoxetine treatment levels and predicted fish plasma concentrations at an exposure pH of 7.96.

Nominal treatment level (µg/L)	Predicted $F_{ss}PC$ (µg/L)
0.3	1.35
3	13.5
10	45
20	90
50	225

Treatment levels were also set such that the lowest treatment level was within an order of magnitude of environmentally realistic concentrations of fluoxetine.

Survival of fish in the exposure tanks was assessed daily. The tiles in the tanks were also checked for eggs at approximately 1300 hours each day. Eggs were enumerated and placed in aerated hatching tanks of treatment water of the same *S*-

fluoxetine concentration as the exposure tanks. After two days, the number of eggs that were fertilized was recorded. Fish were enumerated upon hatching.

P. promelas *Feeding Rate*

Effects of *S*-fluoxetine exposure on *P. promelas* feeding rate was assessed on exposure day 19. Food was withheld from the fish for 24 hours prior to initiation of feeding trials. One randomly selected male and female from each exposure tank were individually introduced into a 12 L plastic arena containing 4 L of dechlorinated tap water. After an acclimation period of 30 minutes, 100 previously frozen adult brine shrimp were introduced into the arena in 30 ml of dechlorinated tap water by pouring over the edge of the arena from behind an opaque curtain. Fish were observed through small slits in the curtain. Latency to first strike was recorded for each fish. Ten minutes after the introduction of the brine shrimp, the fish were removed from the arena and the remaining brine shrimp were enumerated. Feeding rate was recorded as the number of brine shrimp consumed per minute. After completion of feeding trials, fish were returned to their respective treatment tank. Between feeding trials, the water was changed and the plastic arenas were thoroughly rinsed with dechlorinated tap water.

P. promelas *Swimming Behavior*

On exposure day 20, one randomly selected male and female from each replicate aquarium were individually placed into a 12 L plastic arena containing 4 L of dechlorinated tap water. Because four arenas were analyzed at once, the exterior of the arenas were wrapped with opaque white plastic to prevent fish from observing each other. Fish were allowed to acclimate undisturbed for 30 minutes prior to initiation of 30-

minute behavioral trials. Fish movement was tracked using a black and white 0.85 cm Panasonic (Secaucus, NJ, USA) wv-BP330 series CCD digital camera and the software package NOLDUS EthoVision® Pro (Wageningen, Netherlands) with a capture rate of 5.994 samples per second. Five zones were digitally established in each arena using this software, similar to those described in (Kane et al. 2004) (Figure 4.1), and time spent in and movement between these zones was measured. Mean distance between the fish and the center point of the arena throughout each behavioral trial was recorded. Other behavioral endpoints assessed include time spent moving, mean velocity, total distance moved, and turn angle (change in direction between consecutive samples). After completion of behavioral trials, fish were returned to their respective treatment tank. Between behavioral trials, the water was changed and the plastic arenas were thoroughly rinsed with dechlorinated tap water.

P. promelas *Swimming Performance*

Swimming performance was evaluated for two male and two female *P. promelas* from each exposure tank on exposure days 21 and 22, respectively, using a 2 L Brett-type swim tunnel (Brett 1964) immersed in a tank of dechlorinated tap water and procedures previously reported (Brooks 2002). The swim tunnel was composed of a 450 mm long, 75 mm diameter acrylic tube with a 1 mm mesh screen attached to each end. Water was delivered to the tunnel by a 0.5 horsepower centrifugal pump at a maximum speed of 65 cm/s. Fish were individually introduced to the tunnel and allowed to acclimate undisturbed for 5 minutes. Flow rate in the tunnel was assessed using a Marsh-McBirney (Frederick, MD, USA) flow meter. An initial flow rate of 10 cm/s was produced and fish were allowed to swim at this speed for one minute. The flow rate was increased by 10

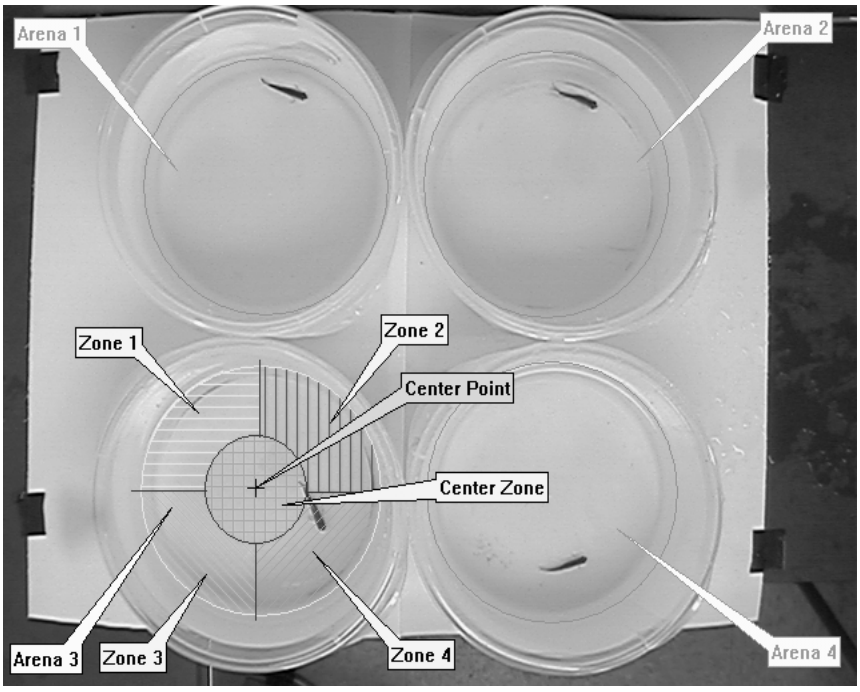

Figure 4.1 Four 12 L plastic arenas used in swimming behavior and feeding rate trials. Digitally defined zones and center point used in swimming behavior trials are shown.

cm/s every minute until the fish was fatigued. Fatigue was defined by the fish stopping swimming and being caught against the rear mesh screen and not resuming swimming when gently prodded (Brett 1964, Brooks 2002). Critical swimming speed was calculated as

$$U_{crit} = u_1 + (t_1/t_2 \times u_2)$$

where u_1 = the highest swimming speed (cm/s) maintained for one minute, u_2 = the speed increment (10 cm/s), t_1 = the time swum at fatigue speed, and t_2 = the time swimming period (60 s) (Brett 1964, Brooks 2002). Critical swimming speed was normalized by individual body length (cm) (Brett 1964, Brooks 2002).

After swimming performance trials, all fish were measured (total length) and sacrificed by decapitation. Blood was collected with a heparinized capillary tube after severing the caudal peduncle. Whole blood was transferred to a microcentrifuge tube and

spun at 1300 x G for 20 minutes. Plasma was collected and immediately placed on dry ice prior to storage at -80 °C. Fish brains were removed and placed in a microcentrifuge tube on dry ice prior to storage at -80 °C for future analysis of brain neurotransmitter concentrations.

Plasma S-*fluoxetine Concentration*

Fish plasma *S*-fluoxetine concentrations were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Immunalysis, Pomona, CA, USA). All commercially available fluoxetine ELISA kits are marketed as qualitative measures only, however, I quantified *S*-fluoxetine concentrations in individual fish by creating a standard curve using dilutions of an analytical fluoxetine standard and a sample size of 5 µL following discussion with technical staff at Immunalysis (Pomona, CA, USA). The standard curve was created using fish blood from an uncontaminated reference site.

Statistical Analyses

Statistical significance of response variables was assigned at $\alpha = 0.05$ for all tests except for the swimming behavior endpoints that were assessed at $\alpha = 0.1$. Statistical analyses were performed using the statistical software package SPSS 13.0 (Chicago, IL, USA). Analyses of all endpoints were performed using parametric one-way analysis of variance (ANOVA) along with a Dunnett's multiple range test. In addition, trends in swimming behavior endpoints were also analyzed by simple linear regression as this approach has been suggested to be a more appropriate method to analyze often highly variable swimming behavior endpoints (Grillitsch et al. 1999).

Results

Water temperature was maintained at a mean (± standard deviation) temperature of 25.2 ± 0.8 °C. Mean pH and DO measurements were 7.96 ± 0.09 and 7.98 ± 1.16, respectively. Mean (± standard deviation) hardness and alkalinity values were 135.0 ± 5.2 and 102.4 ± 8.4 mg/L as $CaCO_3$, respectively. Total residual chlorine levels were < 0.02 mg/L throughout the experiment. Predicted and measured plasma fluoxetine concentrations are shown in Figure 4.2.

No significant differences from controls were observed for egg production, fertilization rate, or hatching success. Both male and female *P. promelas* showed reductions in feeding rate with *S*-fluoxetine treatment (Figure 4.3); however, sex-specific differences in toxicity to this endpoint were observed. The LOEC for male fish was 50µg/L, while the LOEC for females was 10 µg/L. No significant differences were observed in latency to first strike in the feeding trials. Increasing reductions in mean critical swimming speed (U_{crit}) were observed with increasing treatment levels in female fish ($p = 0.38$); however, male fish response was more variable ($p = 0.54$) (Figure 4.4). No statistically significant differences from controls were observed in males or females for this endpoint. Also, no statistically significant differences from controls were observed for any of the swimming behavioral endpoints due to the variable nature of these endpoints (Figures 4.5-4.10). Simple linear regression models for each these endpoints were also not significant in male or female fish. In order to determine if trial duration was the driving factor in the variability in fish responses, the 30-minute behavioral trial data were also analyzed at a trial durations of five minutes. Fish behavioral response at this time was also variable, and no significant differences from controls were observed at this time interval.

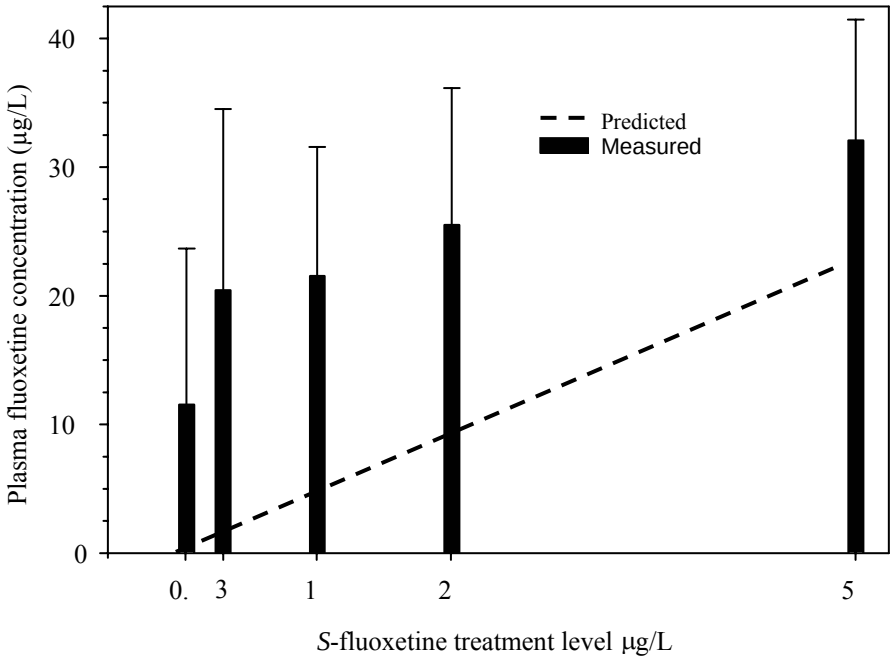

Figure 4.2 Predicted and measured plasma fluoxetine concentrations. Predicted values obtained using the model proposed by Huggett et al. (2003b). Plasma concentrations measured by enzyme-linked immunosorbent assay.

Interestingly, untreated control male fish consistently had markedly lower variation around the mean for a variety of behavioral endpoints than fish exposed to *S*-fluoxetine. Table 4.2 shows the coefficients of variation of the male fish responses in the controls relative to the fluoxetine treatments. This phenomenon was less pronounced in female fish behavioral responses.

Discussion

Because pharmaceuticals are designed to elicit sublethal responses and are generally present in the environment at trace (ng/L) concentrations, acute effects from exposure to this class of contaminant are unlikely (Ankley et al. 2005). Traditional

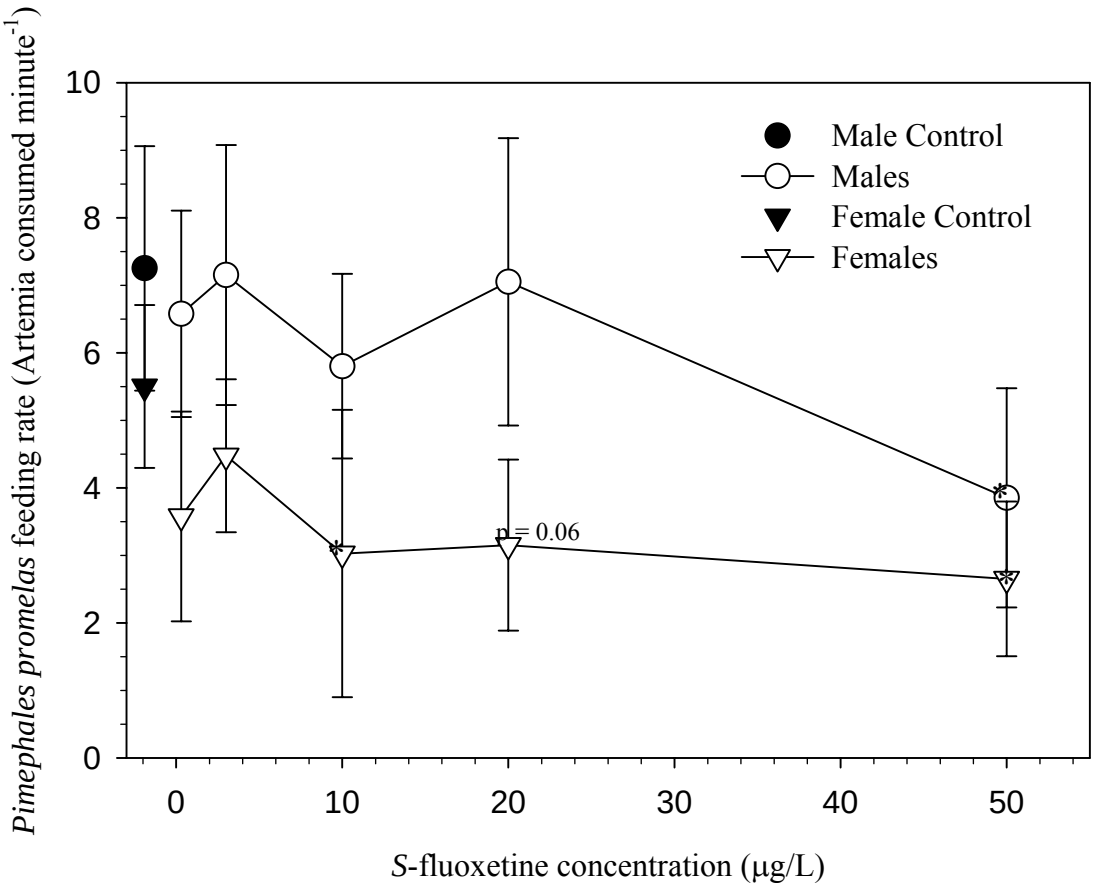

Figure 4.3 Male and female *Pimephales promelas* feeding rate response to 19-day *S*-fluoxetine exposure ($n = 4$; ANOVA with Dunnett's test; error bars are ± 1 standard error; stars indicate a statistically significant difference from the control group; $\alpha = 0.05$).

ecotoxicity testing endpoints (i.e. survival, growth, reproduction) may not be sufficiently sensitive to fully characterize the risk associated with pharmaceutical exposure (Brooks et al. 2003a). The sublethal endpoints of growth and reproduction are widely used and relevant in that perturbations to these endpoints can be directly related to possible population level effects. However, while assessments of these generally-used endpoints allow for comparison of toxicity across compounds with different modes of action, endpoints formulated directly for a known or suspected mode of action for a specific drug may better characterize the risk associated with exposure to that particular compound.

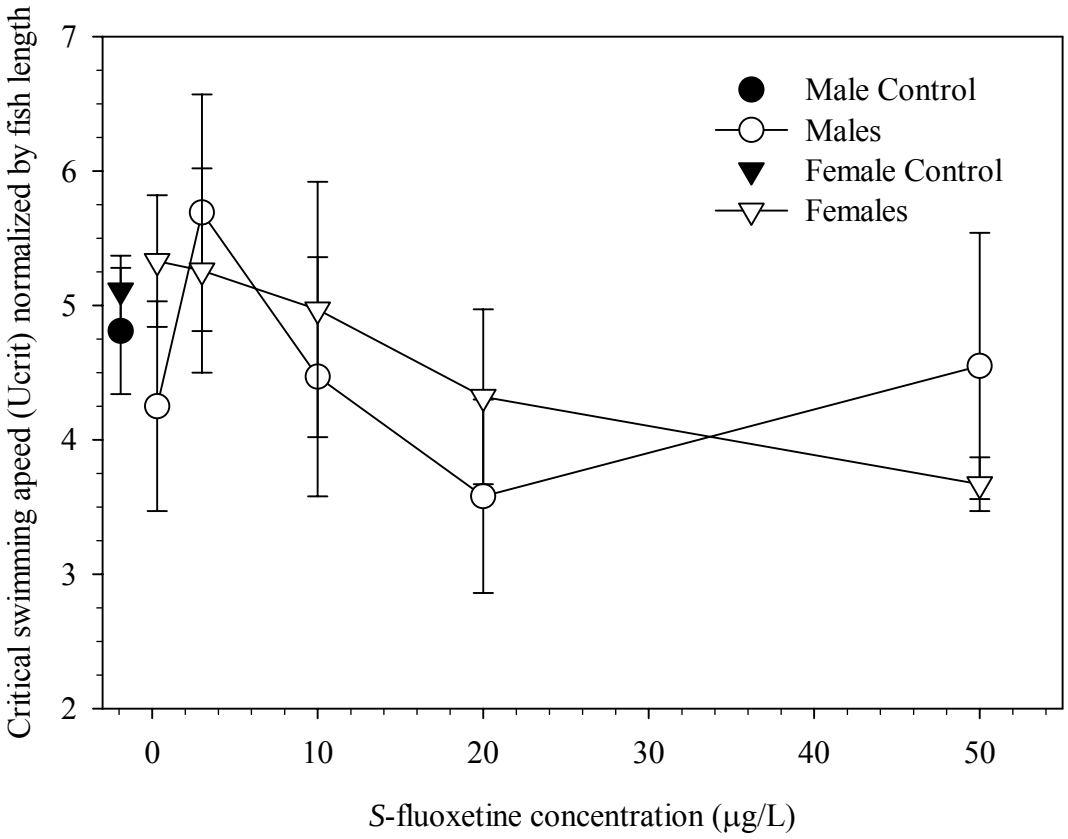

Figure 4.4 Male and female *Pimephales promelas* critical swimming speed (U_{crit}) response to 21 and 22-d, respectively, *S*-fluoxetine exposure (n = 4; error bars are ± 1 standard error)

Table 4.2 Coefficients of variation of behavioral parameters in male *Pimephales promelas* in 30-minute behavioral trials

S-fluoxetine	Coefficients of Variation (%)			
(µg/L)	Distance moved	Velocity	Turn angle	Time spent moving
Control	31.9	31.9	22.2	35.3
0.3	99.4	99.7	39.5	95.1
3	109.7	109.5	44.1	80.8
10	76.7	76.6	72.2	73.3
20	114.0	113.9	42.6	105.0
50	42.5	42.1	42.3	55.4

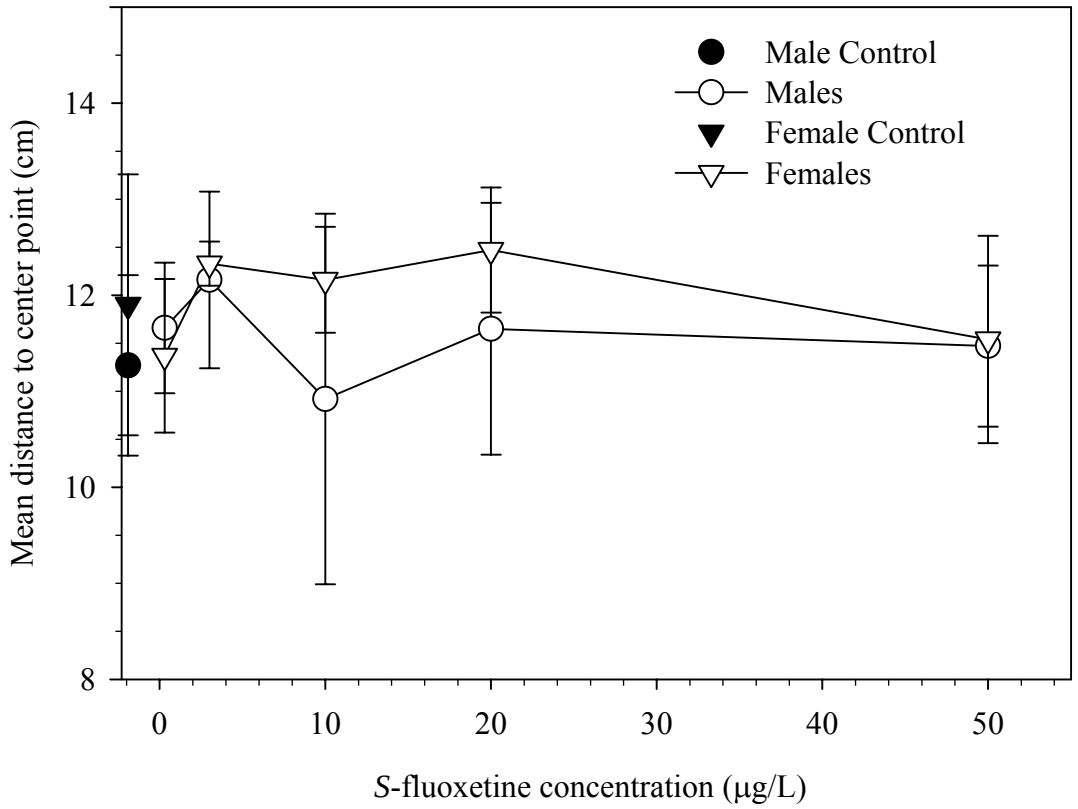

Figure 4.5 Mean distance of fish to center point of arena in 30-minute behavioral trials with male and female *Pimephales promelas* after a 20-day exposure to *S*-fluoxetine (n = 4; error bars are ± 1 standard deviation; ANOVA: male p = 0.77, female p = 0.42; simple linear regression (SLR): male p = 0.97, female p = 0.67)

Mode-of-action-specific biomarkers and endpoints have been recommended for development in non-target aquatic species that are chronically exposed to environmental pharmaceuticals (Daughton and Ternes 1999; Brooks et al. 2003a, Dzialowski et al. 2006). However, in choosing such biomarkers or endpoints, one must be careful not to falsely presuppose that other modes of action are nonexistent or nominal (Daughton and Ternes 1999).

Changes in behaviors such as feeding, such as those observed in the present study, may be the initial response of an organism to a chemical stressor (Fernández-

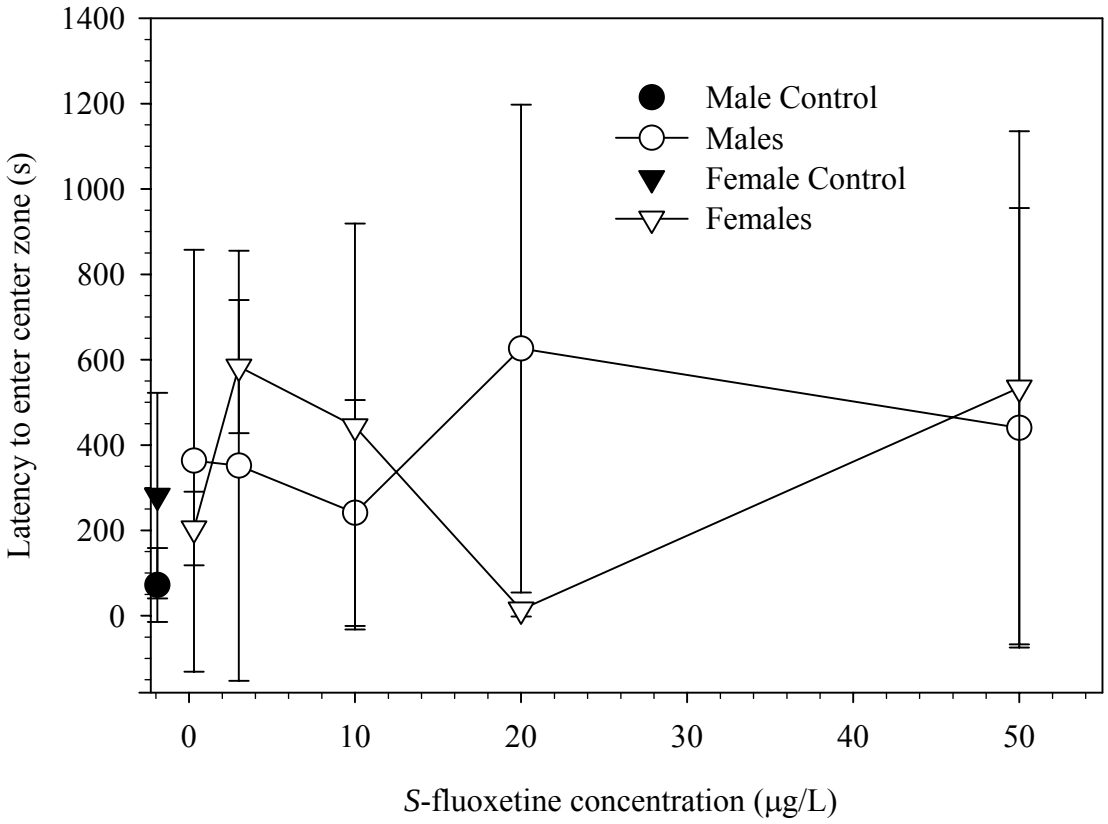

Figure 4.6 Mean latency to enter center zone in behavioral arena of fish in 30-minute behavioral trials with male and female *Pimephales promelas* after a 20-day exposure to S-fluoxetine (n = 4; error bars are ± 1 standard deviation; ANOVA: male p = 0.37, female p = 0.14; SLR: male p = 0.41, female p = 0.61)

Casalderry 1994). Such changes may explain observed reductions in survival, growth, or reproduction (Flickenger et al. 1982; Fernández-Casalderry 1994) and reveal connections between the biochemical, individual, and population levels of biological organization (Weis et al. 2001). Altered predation rates can affect population dynamics of both predator and prey species (Weis et al. 2001).

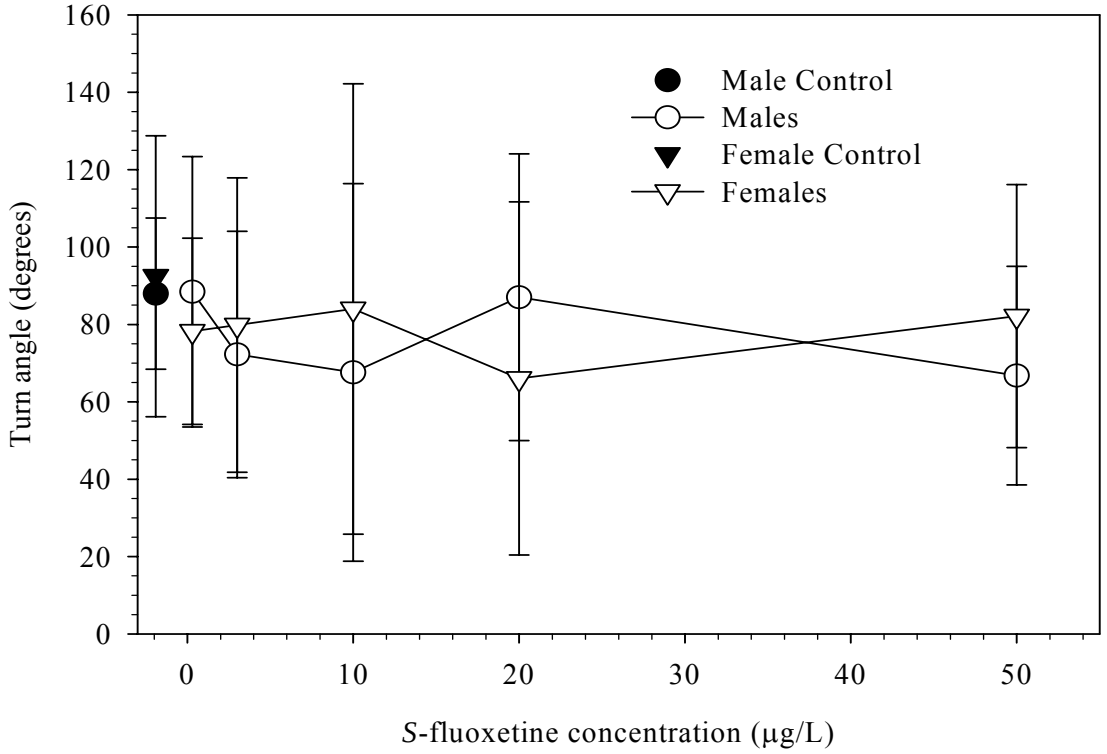

Figure 4.7 Mean turn angle of fish in 30-minute behavioral trials with male and female *Pimephales promelas* after a 20-day exposure to *S*-fluoxetine (n = 4; error bars are ± 1 standard deviation; ANOVA: male p = 0.86, female p = 0.97; SLR: male p = 0.97, female p = 0.36)

The observed feeding rate LOEC for male fish of 50 µg/L *S*-fluoxetine is roughly equivalent to the feeding rate LOEC previously observed in a 7-d exposure with juvenile fathead minnows (Stanley et al. Accepted with revision). However, the LOEC of 10 µg/L for adult females observed in the present study is five-fold lower than that previously reported for juvenile fish (Stanley et al. Accepted with revision). This indicates that the commonly-performed short-term chronic growth and reproduction tests (U.S. EPA 2002b) with juvenile fish are not protective of longer term exposures and exposures with adult fish in all cases. Long-term exposures may be more realistic due to

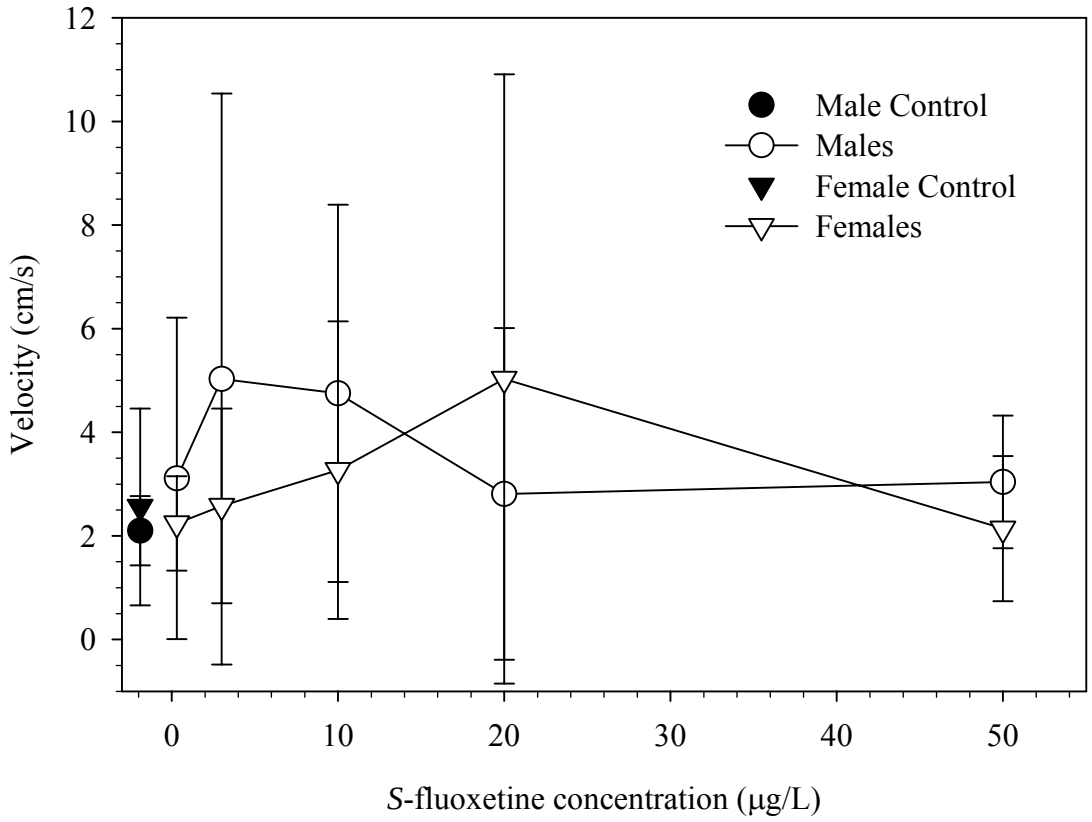

Figure 4.8 Mean velocity of fish in 30-minute behavioral trials with male and female *Pimephales promelas* after a 20-day exposure to *S*-fluoxetine ($n = 4$; error bars are ± 1 standard deviation; ANOVA: male $p = 0.78$, female $p = 0.75$; SLR: male $p = 0.98$, female $p = 0.87$)

the potential of continuous re-introduction or "pseudopersistence" of pharmaceutical compounds in effluent dominated systems.

A possible explanation for the observed sex-specific differences in toxicity of S-fluoxetine is sex-specific differences in the biotransformation of xenobiotics by cytochrome P450s. While P450 levels were not measured in this study, previous researchers have demonstrated markedly lower mixed function oxygenase (MFO) and 7-ethoxyresorufin-*O*-deethylase (EROD) activity as well as concentrations of P450s in reproductively mature female teleosts than in males at specific stages of the reproductive

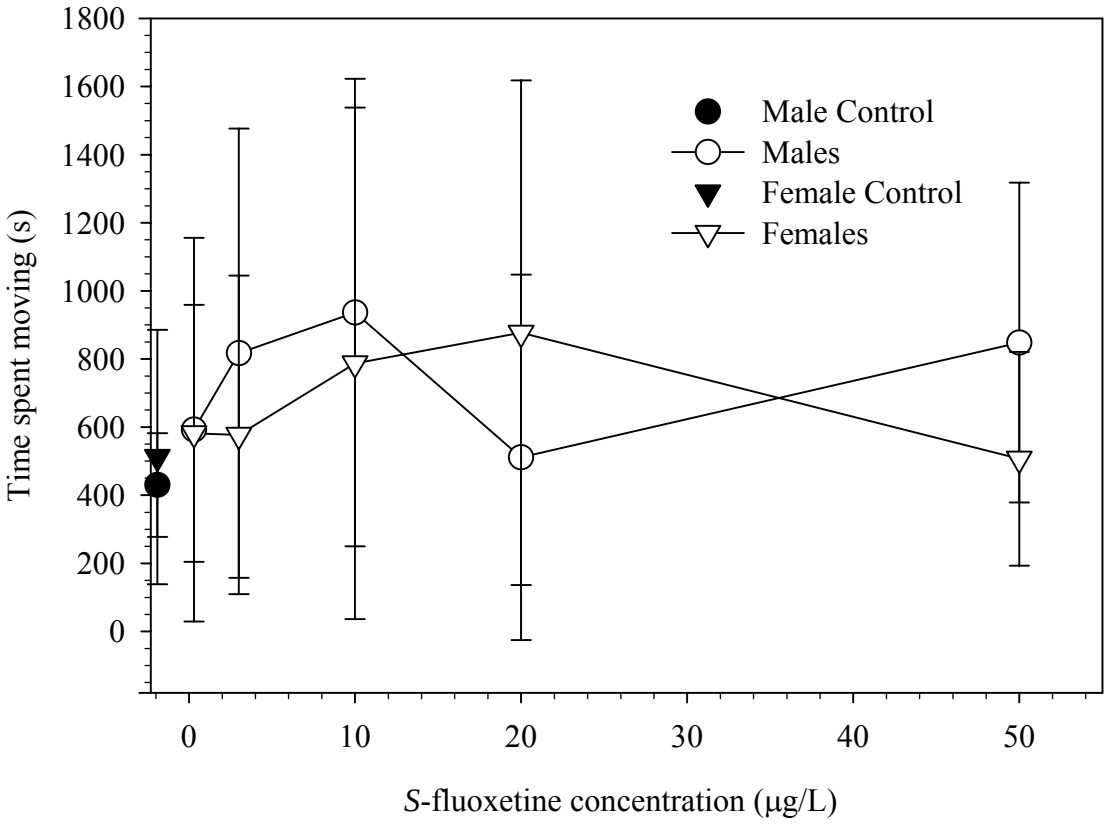

Figure 4.9 Mean time spent moving of fish in 30-minute behavioral trials with male and female *Pimephales promelas* after a 20-day exposure to *S*-fluoxetine (n = 4; error bars are ± 1 standard deviation; ANOVA: male p = 0.72 , female p = 0.89; SLR: male p = 0.68, female p = 0.40)

cycle (Stegeman and Chevion 1980, George et al. 1990, Mathieu et al.1991). The proposed mechanism of these differences is steroidal influence on P450 activity (Hansson et al. 1982). For example, Stegeman et al. (1982) showed hepatic microsomal P450 levels to be depressed by the administration of 17β-estradiol and elevated by the administration of testosterone to immature brook trout (*Salvelinus fontinalis*). Jensen et al. (2001) showed that β-estradiol levels in female *P. promelas* significantly increase, by approximately 100%, one day post-spawn before dropping back to pre-spawn levels.

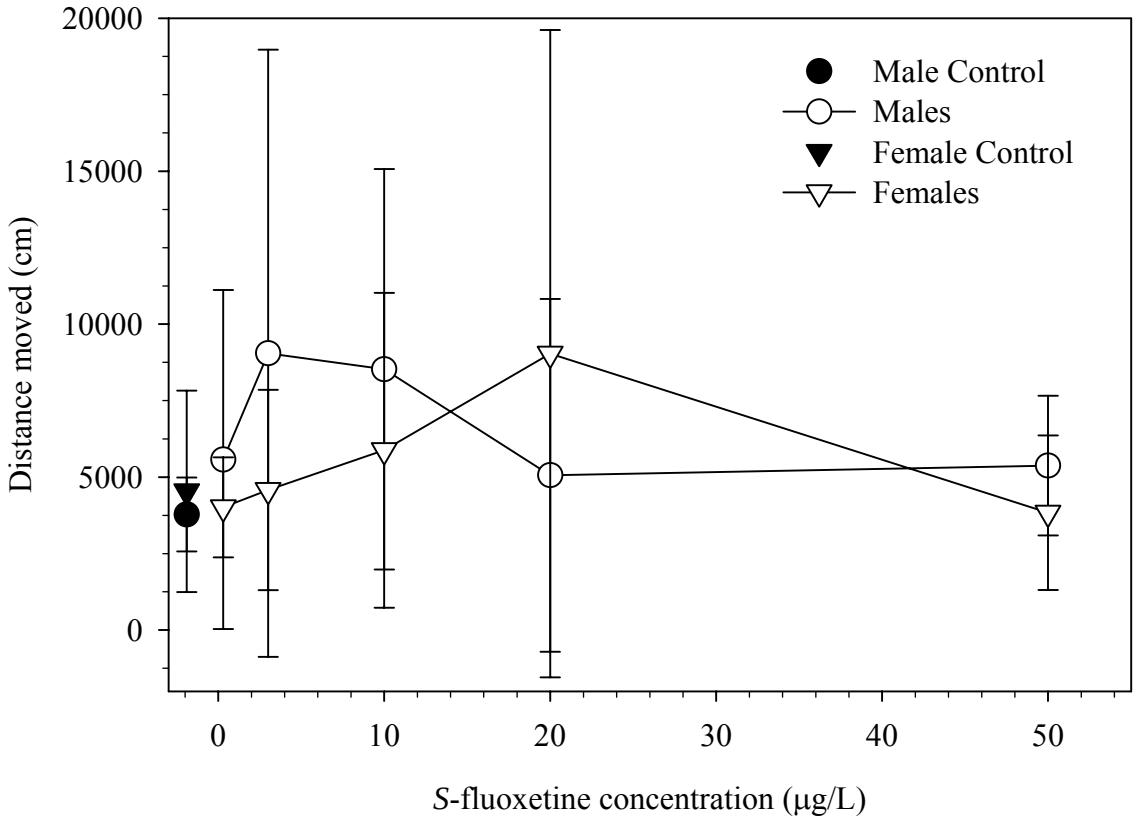

Figure 4.10 Mean distance moved of fish in 30-minute behavioral trials with male and female *Pimephales promelas* after a 20-day exposure to *S*-fluoxetine (n = 4; error bars are ± 1 standard deviation; ANOVA: male p = 0.78, female p = 0.74; SLR: male p = 0.97, female p = 0.87)

Individual female *P. promelas* have been shown to spawn every three to four days, on average, under similar experimental conditions to the present study (Jensen et al. 2001).

The cytochrome P450 isozyme CYP2D6 is the primary enzyme responsible for the demethylation of fluoxetine to its primary active metabolite norfluoxetine in mammals, followed by CYP2C9 and CYP3A4 (Mandrioli et al. 2006). However, further metabolism of norfluoxetine occurs yielding the less active metabolites *p*-trifluoromethylphenol, via CYP2C19 and CYP3A4, and hippuric acid (Mandrioli et al. 2006, Altamura et al. 1994). Conjugation of fluoxetine and norfluoxetine via

glucuronidation has also been demonstrated via radiolabeling studies (Altamura et al. 1994). Because norfluoxetine is as approximately as active an SSRI as the parent drug (Wong et al. 1993, Sweetman 2006), changing the rate of this primary metabolic step due to changes in P450 levels would likely not affect toxicity. However, it is possible that if differential metabolism is the cause of the observed sex-specific differences in the *P. promelas* feeding rate endpoint, that changes in the rate of such secondary metabolic steps could be the cause.

Measured plasma fluoxetine concentrations were well above the levels predicted using the formula given in Huggett et al. (2003b). However, the predicted value was much closer to the mean measured value for the highest treatment level tested than for lower treatment levels (Figure 4.2). One possible explanation for the higher measured concentrations could be that Huggett et al.'s (2003b) model does not take into account dietary exposure to contaminants. It purposely only considers that taken up at the gill as there is little data generated during mammalian testing in the drug development and registration process that could be used to estimate dietary exposure to fish (Huggett et al. 2005). Dietary exposure to *S*-fluoxetine could have occurred in the *P. promelas* exposure either from fluoxetine binding to the *Artemia* fed to the fish or from the fish eating attached algae or waste in the tank that contained bound fluoxetine. Also, because treatment levels in the present study are nominal concentrations, it is possible that *S*-fluoxetine levels in the tanks were actually higher than nominals, and this could have caused the higher than expected plasma concentrations. All measured plasma fluoxetine concentrations were in the range of human therapeutic concentrations, although only treatment levels of 10 µg/L and higher for females and of 50 µg/L for males showed

significant effects. Because I was using an ELISA kit not marketed as a quantitative tool and our measured concentrations had high variability, these observations should be confirmed by more definitive quantitative measures such as HPLC-MS.

The present study is the fist attempt to use a digital tracking system to analyze fish swimming behavioral responses to long-term aqueous exposures of pharmaceuticals at concentrations approaching environmentally realistic levels. High variability of swimming behavioral endpoints was observed, potentially precluding detection of significant effects on the endpoints tested. The increased variability in behavioral responses in male fish exposed to *S*-fluoxetine relative to controls may indicate a response to treatment; however, it is also possible that this is a data artifact as the endpoints this was observed for are interrelated. This response should be tested in future studies. Higher replication may have aided in the detection of effects by increasing statistical power; however, behavioral trials would have had to be significantly shortened in order to analyze the behavior of appreciably more fish than the 48 tested in the daytime hours of a one-day period. The 30-minute trial period is consistent with other studies found in the literature (e.g., Kane et al. 2004); however, there is no standardized procedure. For example, in the experiments surveyed in the literature, duration of digital tracking fish swimming performance studies ranged from two minutes (Brewer et al. 2001) to 48 hours (Winberg et al. 1993).

Studies using similar digital tracking swimming behavioral methods have been used to successfully detect significant swimming impairment relative to controls in fish while exhibiting less variable responses. However, these studies have primarily focused on contaminants known to directly effect neuromuscular control, such as

acetylcholinesterase inhibitors (Brewer et al. 2001, Sandahl et al. 2005), on neurotoxic compounds (Kane et al. 2004), or on contaminants at concentrations approaching acutely toxic levels (Grillitsch et al. 1999). Previous research using digital imaging methods has shown effects of a serotonin reuptake inhibiting pharmaceutical on fish swimming behavior; however, in this study, the pharmaceutical was injected intraperitoneally into the fish immediately prior to analysis of behavior (Winberg et al. 1993). The study by Perreault et al. (2003) noted decreased swimming activity and territorial aggression in a coral reef fish with fluoxetine treatment that was also administered by intraperitoneal injection. However, this study used basic observational techniques and not digital imaging tracking methods. Internal blood pharmaceutical concentrations were not measured in the Winberg et al. (1993) or the Perreault et al. (2003) studies, prohibiting comparisons to the internal fluoxetine concentrations measured in the present study. To my knowledge, no other published study has used behavioral tracking software to quantify the swimming behavior of fathead minnows, preventing variability comparisons with other studies using this species. It is possible that chronic aqueous exposure to low levels of antidepressant pharmaceuticals produces effects on swimming behavior that are too subtle to be measured by the methods employed in this study or that such effects do not occur at the concentrations tested.

Because fate and effects of enantiomers of a compound can significantly differ, independent knowledge of each enantiomer is needed to reduce uncertainty in the assessment of environmental risk of chiral contaminants. The goal of the present study was to provide such data for the enantiomer of fluoxetine previously shown to have the greatest toxicity to fish (Stanley et al. Accepted with revision). Future studies should

continue to provide enantiospecific fate and effect data for all types of chiral contaminants with the goal of increasing accuracy of risk assessment and the usefulness of regulations for such compounds.

CHAPTER FIVE

Existing Regulatory Guidance for Chiral Compounds and Recommendations for Chiral Research and Risk Assessment

In a 1989 book on chiral chromatographic separations Ariëns stated, "Neglect of stereochemistry in the study of drugs, crop protectants, pesticides, and so on, has resulted in massive and wasteful generation of pseudo-scientific nonsense" (Ariëns 1989). While awareness of the environmental consequences of stereochemistry have increased since this paper was written, it is still a widespread practice to ignore enantiospecific differences in fate and effects when estimating risk of chiral contaminants today (Wong 2006). This happens when achiral analytical methods are used to quantitate a chiral compound in the environment or when analyses of toxicity of chiral compounds are performed with a racemate or other mixture of enantiomers instead of individual enantiomers. Without a thorough understanding of enantiospecific fate and effects, unnecessary uncertainty is introduced into the assessment of risk posed by chiral contaminants. For example, see Table 5.1 showing reported enantiospecific differences in toxicity in the peer-reviewed literature including those from the present study.

Most environmental research on chiral contaminants has been limited to legacy organochlorine pesticides (e.g., DDT) and polychlorinated biphenyls (PCBs) that are no longer used in the United States, but whose persistence in the environment keeps them

Table 5.1 Published ecotoxicology enantiospecific effects data

Chiral compound	Test organism	Endpoint	Enantiospecific effect level (µg/L)		Toxicity difference between enantiomers	Reference
Fipronil (insecticide)	*C. dubia*	48-h LC50	(+)-fipronil (-)-fipronil	10.3 31.9	3.1	Konwick et al. 2005
Bifenthrin (insecticide)	*C. dubia*	96-h LC50	(+)-*cis*-bifenthrin (-)-*cis*-bifenthrin	0.076 1.342	9.3	Liu et al. 2005a, Liu et al. 2005b
	D. magna	96-h LC50	(+)-*cis*-bifenthrin (-)-*cis*-bifenthrin	0.081 1.803 0.175	22.3	Liu et al. 2005b
Permethrin (insecticide)	*C. dubia*	96-h LC50	(+)-*cis*-permethrin (-)-*cis*-permethrin	0.156 > 6.0	>38.5	Liu et al. 2005b
			(+)-*trans*-permethrin (-)-*trans*-permethrin	0.197 > 6.0	>30.5	Liu et al. 2005b
	D. magna	96-h LC50	(+)-*cis*-permethrin (-)-*cis*-permethrin	0.388 > 6.0	>15.5	Liu et al. 2005b
			(+)-*trans*-permethrin (-)-*trans*-permethrin	0.307 > 6.0	>19.5	Liu et al. 2005b
Fonofos (insecticide)	*C. dubia*	96-h LC50	(+)-fonofos (-)-fonofos	2.24 0.15	14.9	Liu et al. 2005b
	D. magna	96-h LC50	(+)-fonofos (-)-fonofos	3.45 0.23	15	Liu et al. 2005b

Table 5.1 (continued) Published ecotoxicology enantiospecific effects data

Chiral compound	Test organism	Endpoint	Enantiospecific effect level (µg/L)		Toxicity difference between enantiomers	Reference
Profenofos (insecticide)	*C. dubia*	96-h LC50	(+)-profenofos (-)-profenofos	1.68 0.14	12	Liu et al. 2005b
	D. magna	96-h LC50	(+)-profenofos (-)-profenofos	2.32 0.35	6.6	Liu et al. 2005b
Leptophos (insecticide)	*D. pulex*	24-h LC50	(+)-leptophos (-)-leptophos	0.039 0.800	20.5	Yen et al. 2003
Fenamiphos (insecticide)	*D. pulex*	24-h LC50	(+)-fenamiphos (-)-fenamiphos	1.6 6.1	3.8	Wang et al. 2004
Propranolol (pharmaceutical)	*D. magna*	21-day Immobilization LOEC	(S)-propranolol (R)-propranolol	>869 409.3	>2.1	Stanley et al. 2006
Propranolol (pharmaceutical)	*P. promelas*	7-day growth LOEC	(S)-propranolol (R)-propranolol	>463.6 134.4	>3.4	Stanley et al. 2006
Fluoxetine (pharmaceutical)	*P. promelas*	7-day survival LOEC	(S)-fluoxetine (R)-fluoxetine	101 170	1.7	Stanley et al. Accepted with revisions
Fluoxetine (pharmaceutical)	*P. promelas*	7-day growth LOEC	(S)-fluoxetine (R)-fluoxetine	51 170	3.3	Stanley et al. Accepted with revisions
Fluoxetine (pharmaceutical)	*P. promelas*	7-day feeding rate LOEC	(S)-fluoxetine (R)-fluoxetine	51 170	3.3	Stanley et al. Accepted with revisions

relevant. However, many current use pesticides (e.g., pyrethroids and organophosphate pesticides) as well as so-called "emerging" contaminants such as brominated flame-retardants and pharmaceutical and personal care products are chiral, and there is increasing awareness of enantiospecific differences in fate and effects of such compounds.

Regulation of Chiral Pharmaceuticals in the United States

Due to the direct link to human health, the pharmaceutical industry and its regulators have long been aware of enantiospecific effects of chiral drugs. In May of 1992, The United States Food and Drug Administration's (U.S. FDA) Center for Drug Evaluation and Research issued guidance on the development of new chiral drugs (U.S. FDA 1992). However, it should be noted that such guidance documents merely represent U.S. FDA's current thoughts on a topic, and are not legally binding. This document suggests that manufacturers should use appropriate manufacturing and control procedures in order to assure the stereoisomeric composition of a product and include this information in their new drug application package. It also states that the pharmacokinetics of each enantiomer should be assessed individually and assumed to be different until shown otherwise. However, when it comes to assessing toxicity, U.S. FDA's guidance states that it is ordinarily sufficient to carry out toxicity studies on the racemate only unless toxicity other than that predicted from the pharmacological properties of the drug occurs at relatively low concentrations when compared to those used in clinical trials. If this unexpected toxicity occurs, then U.S. FDA suggests that the same study be repeated with the individual enantiomers to determine if only one of the enantiomers was responsible for the toxic effects observed. If toxicity of significant

concern can be eliminated by the development of a single enantiomer formulation of the drug, the document suggests that it would in general be desirable to do so.

While U.S. FDA's guidance does require investigation of enantiospecific differences in drug pharmacokinetics (i.e., absorption, distribution, metabolism, and elimination), it does not require clear identification of enantiospecific differences in pharmacodynamics short of significant toxicity. Pharmacodynamics is the study of the mode of action and effects a pharmaceutical has on an organism. This omission is problematic in that it does not facilitate prediction of mode-of-action targeted endpoints of concern in non-target organisms. Enantiomers of the same drug can have different pharmacodynamic profiles. For example, the *R*-enantiomer of the β-blocker pharmaceutical sotalol has both β-blocker and antiarrhythmic activities while the *S*-enantiomer has antiarrhythmic properties but lacks β-adrenergic antagonism (McConathy and Owens 2003). Another example would be how *S*-propranolol is by far the most active β-blocker (Howe and Shanks 1966, Barrett and Cullum 1968), while the *R*-enantiomer is responsible for propranolol's membrane stabilizing effect (Kim et al. 2003).

The 1992 guidance document discussed above mentions nothing about environmental concerns of pharmaceuticals. The U.S. FDA addressed this in 1998 when they published another guidance document on environmental assessments that were required to be included in new human drug and biologics applications (U.S. FDA 1998). This document provides instructions for the prediction of expected environmental concentrations as well as a tiered process for the assessment of ecotoxicological effects; however, it does not directly address the issue of stereochemistry of pharmaceuticals in

the environment. The research presented above has demonstrated significant enantiospecific differences in sublethal effects of propranolol (Stanley et al. 2006) and fluoxetine (Stanley et al. Accepted with revision) to highly ecologically relevant endpoints such as growth, reproduction, and feeding rate. This, when coupled with research demonstrating enantiospecific differences in environmental fate of pharmaceuticals such as that presented in Nikolai et al. (2006) and Fono and Sedlak (2004), clearly show that stereochemistry should be considered in environmental assessments of pharmaceuticals.

Regulation of Chiral Pesticides in the United States

Limited interim guidance also exists for chiral pesticides. The Environmental Fate and Effects Division of the U.S. Environmental Protection Agency (U.S. EPA) (2000) issued an interim approach for the determination of data requirements for registration of non-racemic mixtures of chiral pesticides that were previously registered in the racemic form. This short guidance document focuses on the comparison of fate and effects of already registered racemic pesticide mixtures with new enantiomer-enriched formulations and simply calls for more data when significant differences are observed in either. However, what is considered to be significantly different is not defined in this document. The document states that it should be considered interim guidance because the minimal data sets required by this policy may change in the future due to the lack of knowledge about the potentially different behaviors of individual enantiomers and racemic versus non-racemic mixtures.

This guidance is admirable in that it does require basic acute and chronic tests of differences in toxicity between racemic and non-racemic mixtures of chiral pesticides

with avian (or mammalian), aquatic, and nontarget plant species as well as a basic aerobic soil metabolism study. However, it is lacking in that it does not require testing of fate and effects of individual enantiomers of chiral pesticides. Testing an enantiomer-enriched mixture, a mixture whose enantiomer ratio (ER) has been shifted so that the more active enantiomer is in abundance, still is treating a mixture of enantiomers as a single compound and leaves uncertainty as to the enantiospecific fate and effects of the individual isomers. ER is simply the ratio of the concentrations of one enantiomer to another (E1:E2). In chiral analytical measurements it is customary for E1 to refer to the first eluting enantiomer and E2 the second (Kallenborn and Hühnerfuss 2001). Further, this guidance does not deal with enantiospecific differences of pesticides that are already on the market and in the environment. This lack of information about individual enantiomers provides no foundation of knowledge to aid in enantiospecific risk assessment once the enantiomer ratio has shifted in the environment.

Challenges to Risk Assessment of Chiral Compounds – Fate

There are many factors that complicate the prediction of the environmental fate and thus the environmental risk of chiral compounds in the environment. For example, changes in the make up of the resident microbial community can shift or even reverse the enantiomer fraction of a chiral contaminant in a receiving system (Garrison 2006). Alternately, microbial transformations can be non-enantioselective (Padma et al. 2003). Additionally, interconversion between enantiomers, called enantiomerization, can occur through natural biodegradation processes. Buser and Müller (1998) demonstrated this through laboratory studies on the enantiomerization of the herbicides mecoprop and dichloroprop using natural microbial assemblages. Abiotic factors can also indirectly

affect environmental ERs. Maruya et al. (2005) showed that temperature effected enantioselectivity of toxaphene, a nonsystemic biocide, elimination in mummichogs (*Fundulus heteroclitus*).

Wedyan and Preston (2005) demonstrated that some sediments can exhibit enantioselective sorption of chiral compounds. While the authors were not able to pinpoint the primary cause of enantiomer selectivity in this study, by far the most pronounced enantioselective sorption was observed for sediments that had all organic material removed by ashing. This observation, along with some direct study using quartz and the clay minerals montmorillonite and kaolin indicated a possible mineral enantioselectivity component of this phenomenon. Further studies of enantioselectivity of whole natural sediments are needed because differential sorption of contaminants has the potential to affect exposure regimes for organisms living in different microhabitats or occupying differing ecological niches (Belden et al. 2007).

Another factor that complicates assessment of fate of chiral compounds is the fact that compounds can have multiple stereogenic centers. This can result in many more different enantiomer configurations than the two observed for the chiral compounds used in the present research, making assessments of fate even more difficult.

Challenges to Risk Assessment of Chiral Compounds – Effects

Currently, there is much less data available on enantiospecific effects than on enantiospecific fate of chiral contaminants. This lack of effect data is perhaps the biggest impediment to more comprehensive risk assessments of chiral compounds. Because of the stereospecific nature of biological receptors, different enantiomers of a chiral compound can cause different biological effects. For example, one enantiomer can cause

an effect while the other is inactive, or both enantiomers can cause variable levels of the same effect. However, the difference in effects caused by enantiomers can be more than just a question of magnitude. Enantiomers can have completely different modes of action and effects. For example, one enantiomer of a pharmaceutical can cause a desirable effect while the other one is toxic. Probably, the widest known example of this is the pharmaceutical thalidomide that was prescribed in Europe in the early 1960s. This drug was prescribed as a sedative in the racemic form; however, one of the enantiomers was found to cause profound limb malformations in the developing fetus when the drug was given to expectant mothers (Kallenborn and Hühnerfuss 2001). Further, interactions between enantiomers of a drug have been shown to sometimes produce unexpected pharmacodynamic and pharmacokinetic profiles (Mehvar and Brocks 2001). Also, research exists showing greater deleterious effects, including carcinogenicity, due to the racemic mixture than for either of the individual enantiomers in some cases (Ali et al. 2005, Stanley et al. 2006). This may be due to the catalytic properties of one enantiomer with respect to another (Ali et al. 2005). Because of the potential for non-additivity in response and the often-different modes of action between enantiomers, one cannot simply use an ER to predict effects of a mixture of enantiomers in a proportional manner.

While quantitative structural activity relationships (QSARs) can take into account three-dimensional configuration, and thus chirality (Kovatcheva et al. 2005), to date, studies demonstrating reliable prediction of enantiospecific toxicity to non-target organisms cannot be found in the literature. Recently, Garrison (2006) of the U.S. EPA suggested that developing a capability to predict enantioselectivity should be the ultimate goal of enantiospecific research as this would allow manufacturers to develop more

single or enriched-enantiomer formulations that would lead to a reduction in the overall quantity of chemicals introduced into the environment. However, QSAR modeling relationships of structure to toxicological response will need to account for mode of action specific responses, which are typically not identified when traditional QSAR approaches have been applied to pharmaceuticals in the environment (Sanderson et al. 2003).

Consequences of Ignoring Stereochemistry in Ecological Risk Assessments

Ignoring enantiospecific differences in fate and effects for chiral compounds by performing an ecological risk assessment (ERA) on a chiral compound as if it were not chiral will lead to unnecessary uncertainty being introduced into the assessment of risk. For example, if achiral analytical methods are used to assess exposure, enantiospecific differences in toxicity and biodegradation exist, and the more toxic enantiomer is preferentially degraded, then toxicity, and thus risk, will be underestimated (Wong 2006). Conversely, if the less toxic enantiomer is preferentially degraded risk will be overestimated. Such inaccuracies have the potential to lead to either overly stringent environmental regulations or insufficient environmental protection. Both of these scenarios would unnecessarily inflate costs associated with environmental remediation and restoration efforts.

Proposed Scheme to Incorporate Chirality into the Environmental Risk Assessment Process

Figure 5.1 describes considerations for risk assessment given different scenarios of enantiospecific differences in fate and/or effects or neither. If only differences in environmental fate exist without differences in effects, then changing the ER will not

result in differential effects to an organism (Liu et al. 2005). Also, if only differences in ecological effects exist without differences in environmental fate, then the cumulative toxicity of the two enantiomers can be estimated from the ER using one of the two mixture paradigms described in the following section, concentration addition or independent action, given the mode of action of toxicity of each enantiomer is known. If enantiospecific differences in fate and effects are known to exist, then the two enantiomers must be considered separate compounds in the estimation of risk. Chirality of contaminants should be considered early in the ERA process.

A proposed decision tree for deciding how to proceed with ERAs of chiral compounds is presented in figure 5.2. The process begins with a determination of whether or not a chiral compound is distributed in a single enantiomer form or as a mixture of enantiomers. If a compound is distributed as a single enantiomer and does not undergo environmental interconversion between enantiomers, called enantiomerization, then an ERA can proceed by traditional ERA methods. If the compound does undergo environmental enantiomerization, then a mixture of enantiomers will result that must be treated as such. If a compound is distributed as a mixture of enantiomers, including racemic or enantiomer enriched formulations, then in order to know how to proceed with an ERA for this compound, one must know whether or not enantiospecific differences in fate and effects exist for a compound. Data on the occurrence or lack thereof of enantiospecific differences in fate and effects must be known in order to complete an ERA that takes into account stereochemistry. If this information is not known, then it should be obtained by experimentation.

Enantiomers as Components of a Mixture in Ecological Risk Assessment

Enantiomers are highly likely to coexist in the environment with each other if they are distributed in racemic or enantiomer-enriched mixtures or if enantiomerization occurs. Therefore, performing ERAs on each individual enantiomers of such a mixture while neglecting the cumulative toxicity and/or other potential interactions between the two is also not appropriate. Because enantiomers are likely to be found together and may have different toxicodynamic and toxicokinetic profiles, considerations of mixture toxicity in the estimations of risk will apply. Enantiomers may only differ in the magnitude of response of the same mode of toxic action. In this case, mixture toxicity can be assessed using concentration addition methods such as toxic units (TUs). TUs is a method to predict mixture toxicity when additive toxicity exists between the members of the mixture. Each chemical in the mixture's concentration is divided by a benchmark of toxicity and the values for each chemical are summed. For example, the formula for TUs for a two component mixture is:

$$TU = [A]/LC(X) + [B]/LC(X)$$

where [A] = the concentration of contaminant "A", [B] = the concentration of contaminant "B", and LC(X) = the toxicity benchmark that is being used, commonly the50% lethal concentration or LC50. The contaminants "A" and "B" in this equation could be two different enantiomers. If the summed values equal one or greater, this indicates the potential for toxicity greater than or equal to the chosen benchmark. For example, in a mixture of two enantiomers, if the *S*-enantiomer's LC50 is 10 mg/L and it is present in the mixture at 7 mg/L and the *R*-enantiomer's LC50 is 12 mg/L and is present in the mixture at a concentration of 6 mg/L, the toxic units would equal 1.2, see

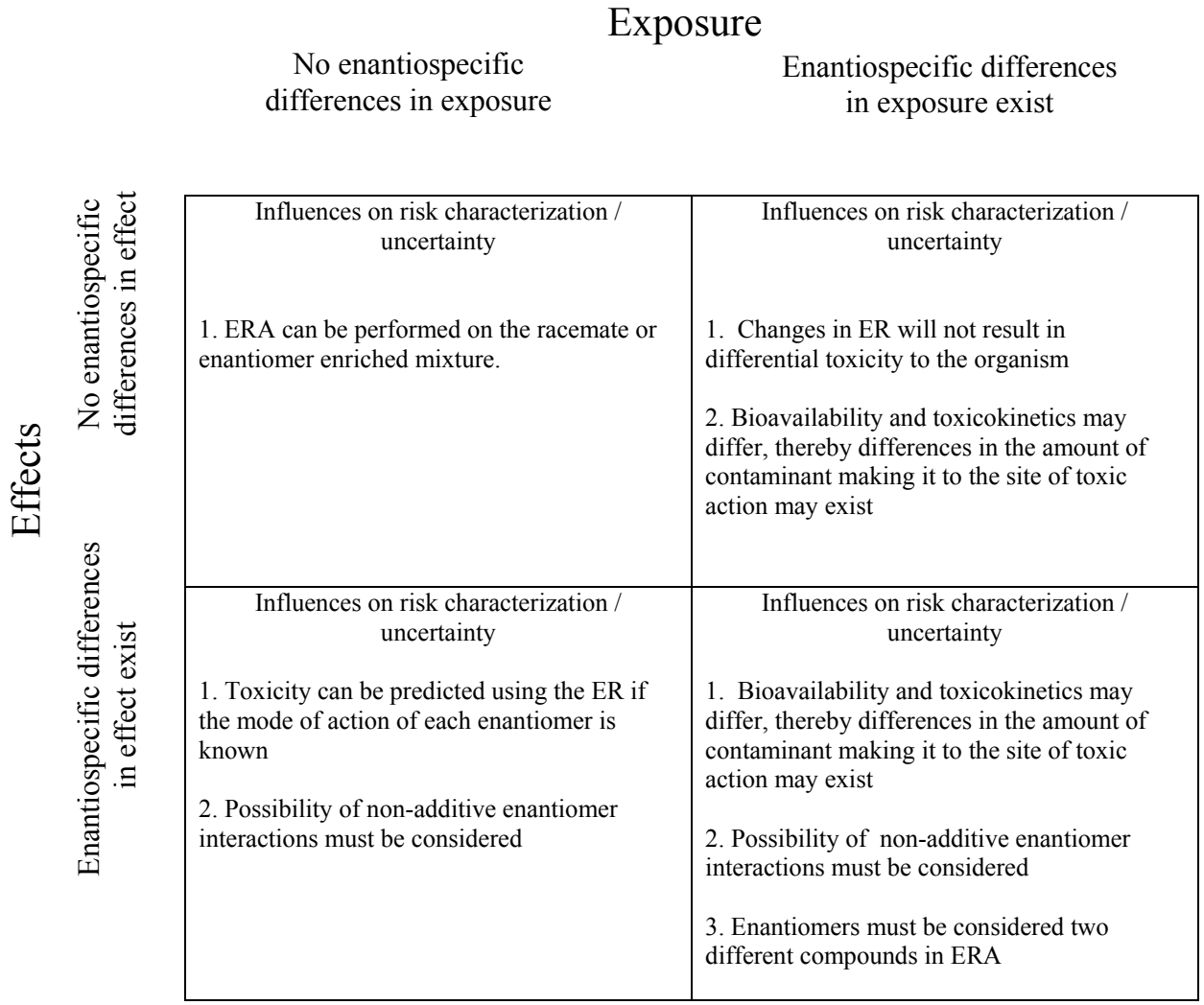

Figure 5.1 Influences on risk characterization/uncertainty because of enantiospecific differences in fate and effects or the lack thereof between enantiomers of a chiral contaminant

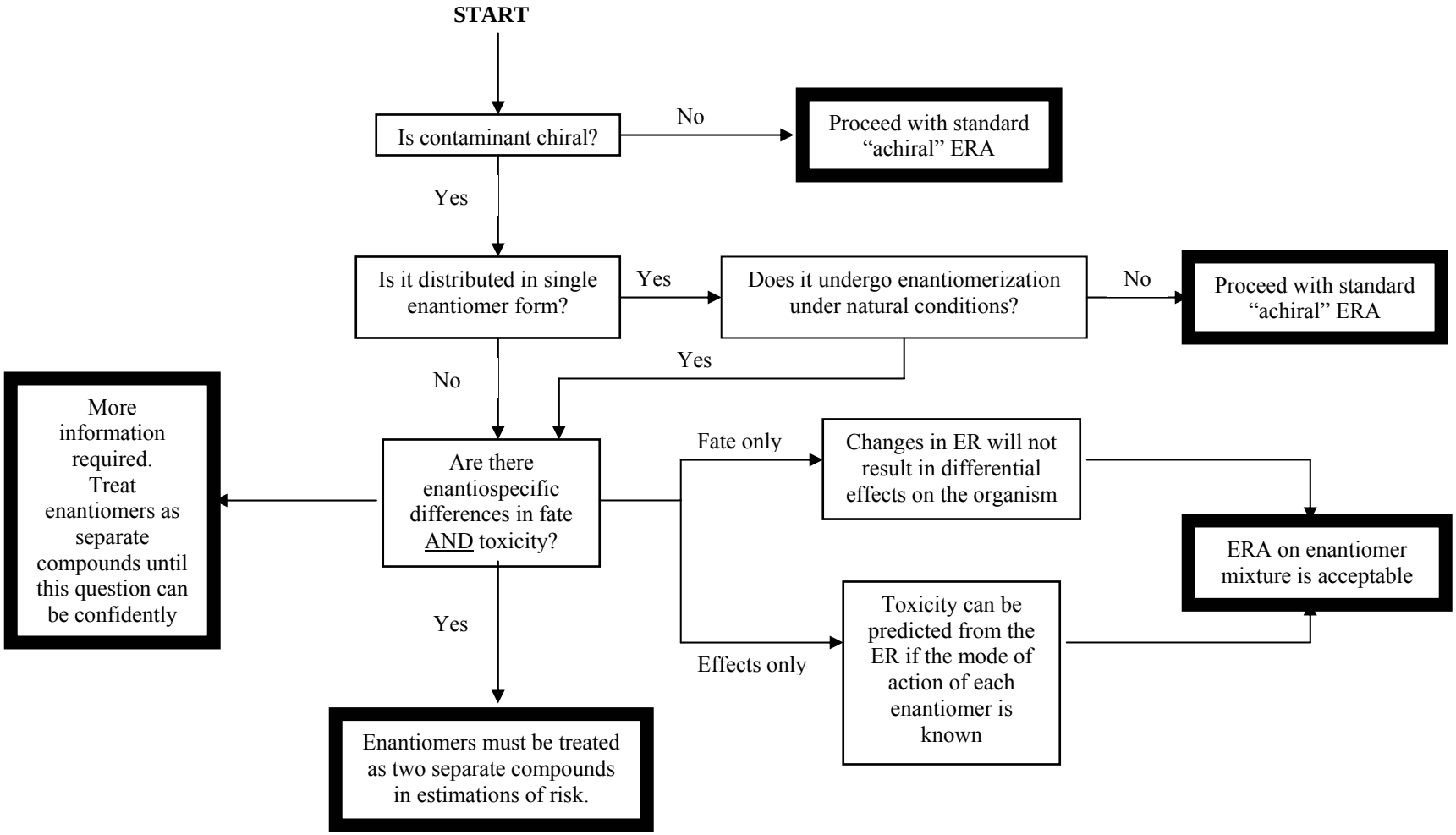

Figure 5.2 Proposed decision tree for ecological risk assessments (ERAs) of chiral contaminants.

calculation below, indicating a mixture that is predicted to kill greater than 50% of exposed organisms.

$$(7/10) + (6/12) = 1.2$$

However, it should be noted that the traditional toxic unit approach of dividing the environmental concentrations by an acutely toxic amount such as the LC50 is not appropriate for all compounds. An example is pharmaceutical contaminants that are specifically designed to be biologically active but not acutely toxic and may have exceptionally large acute to chronic ratios (ACR). The ACR is normally calculated by dividing the acute LC50 by the chronic no observable effect concentration (NOEC). ACR values of ≤10 are typical and values >40 are rare; however, pharmaceutical compounds can have ACRs that are in the tens to hundreds of thousands (Ankley et al. 2005). In addition, it is important to remember that enantiomers can sometimes exhibit relationships other than additivity. An example is the greater than additive toxicity demonstrated by the racemate of benzo[*a*]pyrene 7,8-oxide by Levin et al. (1980).

Because enantiomers can also possess completely different modes of action, assessment of mixture toxicity according to the rules of independent action may also be necessary. Independent action means that the components of a mixture are assumed to behave independently of one another, so the relative effect of one of the enantiomers in a mixture should remain unchanged in the presence of the other one (Cleuvers 2003). The following equation, adapted from Lydy et al. (2004), gives the relationship of independent action for a mixture of two chemicals:

$$\textit{Mixture Effect} = \textit{Effect of S-enantiomer} + \textit{Effect of R-enantiomer} - (\textit{Effect of S-enantiomer} * \textit{Effect of R-enantiomer})$$

Thus, the effect of the two enantiomers is the sum of their individual effects minus the portion of the population for which sensitivities to the two enantiomers overlap (Lydy et al. 2004). Effects are entered as proportions. So, for example, if the S-enantiomer is 30% toxic and the R-enantiomer is 40% toxic, the predicted mixture effect would be 58%.

The fact that knowledge of an individual chemical's mode of toxic action is critical to the understanding of mixture effects involving that chemical (Lydy et al. 2004) again reiterates the need for individual assessments of effect for each component of a mixture of enantiomers. Without knowledge of each component of a mixture's toxicodynamics, ability to predict and understand observed toxicity of mixtures is greatly impaired (Lydy et al. 2004). Thus the need for enantiospecific effect data like that presented above is clear. However, as illustrated in figure 5.1, knowledge of each enantiomer's fate and bioavailability are also required in order to estimate risk of chiral contaminants, as toxicity is a function of the magnitude, duration and frequency of exposure. In addition, just as understanding environmental fate is important, so is understanding toxicokinetics at the organismal level. For example, if one enantiomer is metabolized and/or excreted slower than the other, then this enantiomer will be able to exert an effect at a site of toxic action over a longer duration than its antipode. For example, *S*-fluoxetine is eliminated from the body more slowly than *R*-fluoxetine in mammals, causing this to be the predominant enantiomer present in the plasma (Guo et al. 2002).

The Proposed Enantiomer Hazard Ratio (EHR)

Suter et al. (2000) describe the hazard index (HI) approach, which provides particular utility for screening level ecological risk assessments. HI can be characterized by:

$$HI = \sum(SEC_i)/SBCi)$$

where SEC is the screening exposure concentration, SBC is the screening benchmark concentration such as a No Observable Effect Concentration or a Lowest Observable Effect Concentration and *i* is associated with a specific chemical contaminant. In a screening level retrospective ecological risk assessment, the HI approach essentially follows the TU paradigm where the hazard contributed by individual chemicals are presumed to be additive. If an individual chemical contributes ≥ 0.3 then the chemical is typically retained as a chemical of potential ecological concern for further consideration in a definitive ERA (Parkhurst et a. 1996). The HI conceptually provides a useful approach to screen individual enantiomers in a risk assessment, but is inherently limited by additive ecotoxicological presumptions. Therefore, an approach accounting for enantiomer specific effects is required to characterize hazard of chiral contaminants.

Here I propose a method for assessing relative risk associated with enantiomers of an enantiomer pair is the relative enantiomer hazard ratio (EHR) given by the following equation:

$$EHR = \frac{([MEC_S \text{ or } PEC_S] / [TBC_S])}{([MEC_R \text{ or } PEC_R] / [TBC_R])}$$

where MEC_S and MEC_R are the measured environmental concentrations of the S and R enantiomers, respectively. If reliable prediction of enantiospecific fate is developed in the future that allows for the estimation of individual enantiomer concentration in

environmental compartments, then a predicted environmental concentration (PEC) could also be used in the place of a MEC. TBC_S and TBC_R are benchmark effect concentrations of the individual enantiomers. Because enantiomers can have different modes of action, the TBC concentrations for each enantiomer could describe different endpoints for each enantiomer. Choice of endpoints would be essential to the usefulness of this ratio. The endpoints chosen should be sensitive and have clear implications for organismal and population level health. For example, using induction of a biomarker of exposure as an endpoint for one enantiomer that has not been related to deleterious effects on organismal health while using endpoints such as reduced growth or reproduction as an endpoint for the other enantiomer could skew the EHR value causing a likely inaccurate estimation of relative risk. Also, the EHR would have to be adapted for enantiomer mixtures with more than two enantiomer configurations. The major utility of this approach would likely be in retrospective risk assessments of enantiomer mixtures, although it could also be useful in prospective risk assessments if enantiospecific fate is well understood.

In order to standardize the use of the EHR, the *S*-enantiomer should always be placed in the numerator and the *R*-enantiomer in the denominator. If absolute configuration is not known, then the (+)-enantiomer should be placed in the numerator and the (-)-enantiomer in the denominator. If the resulting ratio is > 1, this would mean that greater risk is expected from the *S*- or (+)-enantiomer. If the ratio is < 1, this would indicate that greater risk is expected from the *R*- or (-)-enantiomer.

Further Recommendations for the Facilitation of Chiral Environmental Risk Assessment

The complications of stereochemistry can introduce unnecessary uncertainty into assessments of risk for chiral compounds. However, defining these complications instead of ignoring them will increase the confidence in risk assessments performed for chiral compounds and thereby increase the confidence with which regulators can govern the use of such compounds. Additional recommended steps to facilitate this process are outlined below.

- Single enantiomer formulations of drugs ands pesticides and other chiral compounds that end up as environmental contaminants should preferentially be manufactured and used whenever possible in order to reduce environmental loadings of contaminants. For example, an United States Environmental Protection Agency (U.S. EPA) study found that if farmers in the United States in 2001 had used *S*-metolachlor, which is ten fold more potent a herbicide than its antipode, instead of the racemic mixture of this compound that the environmental load of this herbicide could have been reduced from approximately 10,000 t to approximately 6,000 t (Kiely et al. 2004, Garrison 2006). However, even if compounds are distributed in the single enantiomer form, their propensity to undergo enantiomerization either in the environment or within an organism must still be considered.

- In order to facilitate risk assessment of chiral compounds, more ecotoxicity data is needed on enantiospecific effects to non-target organisms, especially data from chronic exposures.

- The rapidly advancing fields of proteomics and metabolomics and microarray analysis tools should increasingly be used to measure protein production, gene expression, and production of endogenous metabolites in order to elucidate mechanisms of enantiospecific effects (Garrison 2006, Wong 2006).

- Companies should be required to thoroughly analyze environmental fate and effects of each individual enantiomer of a product containing a mixture of enantiomers for all new products and products currently on the market and make these data publicly available.

- Single enantiomer standards should be made more widely available. This would facilitate much needed enantiospecific fate and effect studies.

- Enantiospecific fate studies should be performed at a variety of environmental conditions and with a variety of natural assemblages of microorganisms

- Enantiospecific effect studies with pharmaceuticals and other industrial chemicals should include sub-lethal endpoints that are targeted towards the suspected modes of action of each enantiomer and most sensitive species group. Acute lethality studies of enantiospecific toxicity alone may not be sufficient for classes of contaminants that subtly modulate biochemical pathways and physiological endpoints of ecological relevance.

APPENDICES

APPENDIX A

Peer-reviewed Journal Article and Accepted Manuscript
Derived from the Present Research

Chapter Two

Stanley JK, Ramirez AJ, Mottaleb M, Chambliss CK, Brooks BW. 2006. Enantiospecific toxicity of the β-blocker propranolol to *Daphnia magna* and *Pimephales promelas*. *Environmental Toxicology and Chemistry* 25(7):1780-1786.

Chapter Three

Stanley JK, Ramirez AJ, Chambliss CK, Brooks BW. *Accepted*. Left-handed versus right-handed ecotoxicology: Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. *Chemosphere*.

APPENDIX B

Copyright Letter

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Phone: 785-843-1235, 800-627-0932
Fax: 785-843-1853
E-Mail: acg@acgpublishing.com

Date: March 2, 2007
To: Jacob Stanley
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From: Crystal Macmillan
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… to appear in your Ph.D. dissertation. (Baylor University).

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BIBLIOGRAPHY

Adams CF, Liley NR, Gorzalka BB. 1996. PCPA increases aggression in male firemouth cichlids. *Pharmacology* 53:328-330.

Aldegunde M, Manceboe M. 2006. Effects of neuropeptide Y on food intake and brain biogenic amines in the rainbow trout (*Oncorhynchus mykiss*). *Peptides* 27:719-727.

Alexander BS, Wood MD. 1987. Stereoselective blockade of central [3H]5-hydroxytryptamine binding to multiple sites (5-HT1A, 5-HT1B, and 5-HT1C) by mianserin and propranolol. *J Pharm Pharmacol* 39:664-666.

Ali I, Aboul-Enein HY. 2004. *Chiral pollutants: Distribution, toxicity, and analysis by chromatography and capillary electrophoresis*. Chichester (UK): John Wiley. 351 p.

Ali I, Aboul-Enein HY, Ghanem A. 2005. Enantioselective toxicity and carcinogenesis. *Curr Pharm Anal* 1:109-125.

Altamura AC, Moro AR, Percudani M. 1994. Clinical pharmacokinetics of fluoxetine. *Clin Parmacokinet* 26:201-214.

[APHA, AWWA, WEF] American Public Health Association, American Water Works Association, Water Environment Foundation. 1998. *Standard methods for the examination of water and wastewater*, 20th ed. Washington (DC): American Public Health Association.

Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20(6):1276-1290.

Ankley GT, Black MC, Garric J, Hutchinson TH, Iguchi T. 2005. A framework for assessing the hazard of pharmaceutical materials to aquatic species. In: Williams RT, editor. *Human pharmaceuticals: Assessing the impacts on aquatic ecosystems*. Pensacola (FL): SETAC Press. p 183-237.

Ariëns EJ. 1989. Racemates–an impediment in the use of drugs and agrochemicals. In: Krstulovic AM, editor. *Chiral separations by HPLC*. Chichester (UK): Ellis Horwood. p 31-68.

Baldessarini RJ. 2001. Drugs and the treatment of psychiatric disorders: Depression and anxiety disorders. In: Hardman JG, Limbird LE, editors. *Goodman & Gillman's The pharmacological basis of therapeutics*. New York (NY): McGraw-Hill Medical Publishing Division. p 447-483.

Barrett AM, Cullum VA. 1968. The biological properties of the optical isomers of propranolol and their effects on cardiac arrhythmias. *Br J Pharmacol* 34:43-55.

Baumann P, Zullino DF, Eap CB. 2002. Enantiomers' potential in psychopharmacology—a critical analysis with special emphasis on the antidepressant escitalopram. *Eur Neuropsychopharm* 12:433-444.

Belden JB, Maul JD, Lydy MJ. 2007. Partitioning and photodegradation of ciprofloxacin in aqueous systems in the presence of organic matter. *Chemosphere* 66:1390-1395.

Björklund HV, Bondestam J, Bylund G. 1990. Residues of oxytetracycline in wild fish and sediments from fish farms. *Aquaculture* 86:359-367.

Blundell JE, Halford JCG. 1998. Serotonin and appetite regulation, implications for the pharmacological treatment of obesity. *CNS Drugs* 9:473-495.

Brett JR. 1964. The respiratory metabolism and swimming performance of young sockeye salmon. *J Fish Res Board Can* 21:1183-1226.

Brewer SK, Little EE, DeLonay AJ, Beauvais SL, Jones SB, Ellersieck MR. 2001. Behavioral dysfunctions correlate to altered physiology in rainbow trout (*Oncorynchus mykiss*) exposed to cholinesterase-inhibiting chemicals. *Arch Environ Contam Toxicol* 40:70-76.

Brodfuehrer PD, Debski EA, O'Gara BA, Friesen WO. 1995. Neuronal control of leech swimming. *J Neurobiol* 27:403-418.

Brooks BW. 2002. Ecotoxicological investigations in effluent-dominated stream mesocosms [dissertation]. Denton (TX): University of North Texas. 148 p.

Brooks BW, Foran CM, Richards SM, Weston J, Turner PK, Stanley JK, Solomon KR, Slattery M, La Point TW. 2003a. Aquatic ecotoxicology of fluoxetine. *Toxicol Lett* 142:169-183.

Brooks BW, Turner PK, Stanley JK, Weston JJ, Glidewell E, Foran CM, Slattery M, La Point TW, Huggett DB. 2003b. Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere* 52(1):135-142.

Brooks BW, Chambliss KC, Stanley JK, Ramirez AJ, Banks K, Johnson RD, Lewis RJ. 2004. Determination of select antidepressants in organisms from a municipal effluent-dominated stream [abstract]. In: Preprints of extended abstracts from ACS national meeting, American Chemical Society, Division of Environmental Chemistry, 44(2): 1351-1355.

Brooks BW, Chambliss CK, Stanley JK, Ramirez A, Banks KE, Johnson RD, Lewis RJ. 2005. Determination of select antidepressants in fish from an effluent-dominated stream. *Environ Toxicol Chem* 24(2):464-469.

Brooks BW, Riley TM, Taylor RD. 2006. Water quality of effluent-dominated ecosystems: ecotoxicological, hydrological, and management considerations. *Hydrobiologia* 556:365-379.

Buser HR, Müller MD. 1998. Occurrence and transformation reactions of chiral and achiral phenoxyalkanoic acid herbicides in lakes and rivers in Switzerland. *Environ Sci Technol* 32(5):626-633.

Caner H, Groner E, Levy L. 2004. Trends in the development of chiral drugs. *Drug Discov Today* 9:105-110.

Capone DG, Weston DP, Miller V, Shoemaker C. 1996. Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. *Aquaculture* 45:55-75.

Cleuvers M. 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicol Lett* 142:185-194.

Cleuvers M. 2005. Initial risk assessment for three β-blockers found in the aquatic environment. *Chemosphere* 59:199-205.

Daughton CG, Ternes TA. 1999. Pharmaceutical and personal care products in the environment: agents of subtle change? *Environ Health Persp* 107(Supplement 6):906-938.

de Pedro N, Pinillos ML, Valenciano AI, Alonso-Bedate M, Delgado MJ. 1998. Inhibitory effect of serotonin on feeding behavior in goldfish: involvement of CRF. *Peptides* 19(3):505-511.

Dryden S, Frankish HM, Wang Q, Pickavance L, Williams G. 1996. The serotonergic agent fluoxetine reduces neuropeptide Y levels and the neuropeptide Y secretion in the hypothalamus of lean and obese rats. *Neuroscience* 72(2):557-566.

Dzialowski EM, Turner PK, Brooks BW. 2006. Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*. *Arch Environ Contam Toxicol* 50:503-510.

Evans DH. 1993. *The Physiology of Fishes*. Ann Arbor (MI): CRC Press.

Fernández-Casalderrey A, Ferrando MD, Andreu-Moliner E. 1994. Effects of sublethal concentrations of pesticides on the feeding behavior of *Daphnia magna*. *Ecotox Environ Safe* 27:82-89.

Ferrari B, Mons R, Vollat B, Fraysse B, Paxéus N, Lo Giudice R, Pollio A, Garric J. 2004. Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environ Toxicol Chem* 23:1344-1354.

Ferriere F, Khan NA, Troutaud D, Deschaux P. 1996. Serotonin modulation of lymphocyte proliferation via 5-HT1A receptors in rainbow trout (*Oncorhynchus mykiss*). *Dev Comp Immunol* 20(4):273-283.

Fingerman SW. 1976. Circadian rhythms of brain 5-hydroxytryptamine and swimming activity in the teleost, *Fundulus grandis*. *Comp Biocem Phys C* 54(1):49-53.

Fitzsimmons PN, Fenandez JD, Hoffman AD, Butterworth BC, Nichols JW. 2001. Branchial elimination of superhydrophobic organic compounds by rainbow trout (Oncorhynchus mykiss). *Aquat Toxicol* 55:23-34.

Flaherty CM, Dodson SI. 2005. Effects of pharmaceuticals on *Daphnia* survival, growth, and reproduction. *Chemosphere* 61:200-207.

Fong PP. 2001. Antidepressants in aquatic organisms: A wide range of effects. In: Daughton CG, Jones-Lepp TL, editors. *Pharmaceuticals and personal care products in the environment: Scientific and regulatory issues.* Washington (DC): American Chemical Society. p 264-281.

Fono L, Sedlak DL. 2004. Use of the chiral pharmaceutical propranolol to identify sewage discharges into surface waters. *Environ Sci Technol* 39:9244-9252.

Fraysse B, Garric J. 2005. Prediction and experimental validation of acute toxicity of β-blockers in *Ceriodaphnia dubia*. *Environ Toxicol Chem* 24:2470-2476.

Furlong ET, Kinney CA, Ferrer I, Werner SL, Cahill JD, Ratterman G. 2004. Pharmaceutical and personal care products in solids: Analysis and field results for sediment, soil, and biosolid samples. In: Proceedings of the 228th American Chemical Society National Meeting, Philadelphia (PA).

Gamperl A, Wilkinson M, Boutilier R. 1994. β-adrenoreceptor in the trout (*Oncorhynchus mykiss*) heart: characterization, quantification, and effects of repeated catecholamine exposure. *Gen Comp Endocr* 95:259-272.

Garrison AW. 2006. Probing the enantioselectivity of chiral pesticides. *Environ Sci Technol* 40(1):16-23.

George S, Young P, Leaver M, Clarke D. 1990. Activities of pollutant metabolising and detoxication systems in the liver of the plaice, *Pleuronectes platessa*: Sex and seasonal variations in non-induced fish. *Comp Biochem Phys C* 96:185-192.

Glassmeyer ST, Furlong ET, Kolpin DW, Cahill JD, Zaugg SD, Werner SL, Meyer MT, Kryak DD. 2005. Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. *Environ Sci Technol* 39(14):5157-5169.

Grillitsch B, Vogl C, Wytek R. 1999. Qualification of spontaneous undirected locomotor behavior of fish for sublethal toxicity testing. Part II. Variability of measurement parameters under toxicant-induced stress. *Environ Toxicol Chem* 18(12):2743-2750.

Guo X, Fukushima T, Li F, Imai K. 2002. Determination of fluoxetine enantiomers in rat plasma by pre-column fluorescence derivitization and column-switching high-performance liquid chromatography. *Analyst* 127:480-484.

Halford JCG, Blundell JE. 1996. Metergoline antagonizes fluoxetine-induced suppression of food intake but not changes in the behavioural satiety sequence. *Pharmacol Biochem Be* 54(4):745-751.

Hansson T, Förlin L, Rafter J, Gustafsson JA. 1982. Regulation of hepatic steroid and xenobiotics metabolism in fish. In: Hietanen E, et al. editors. *Cytochrome P-450, biochemistry, biophysics and environmental implications*. New York (NY): Elsevier Biomedical Press. p 217-224.

Heberer T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicol Lett* 131:5-17.

Hegeman WJM, Laane RWPM. 2002. Enantiomeric enrichment of chiral pesticides in the environment. *Rev Environ Contam Toxicol* 173: 85-116.

Hemming JM, Turner PK, Brooks BW, Waller WT, La Point TW. 2002. Assessment of toxicity reduction in wastewater effluent flowing through a treatment wetland using *Pimephales promelas*, *Ceriodaphnia dubia*, and *Vibrio fischeri*. *Arch Environ Contam Toxicol* 42:9-16.

Howe R, Shanks RG. 1966. Optical isomers of propranolol. *Nature* 210:1336-1338.

Huber R, Smith K, Delago A, Isakkson K, Kravitz EA. 1997. Serotonin and aggressive motivation in crustaceans: altering the decision to retreat. *Proc Natl Acad Sci USA* 94:5939-5942.

Huggett DB, Brooks BW, Peterson B, Foran CM, Schlenk D. 2002. Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (β-blockers) on aquatic organisms. *Arch Environ Contam Toxicol* 43:229-235.

Huggett DB, Khan IA, Foran CM, Schlenk D. 2003a. Determination of beta-adrenergic receptor blocking pharmaceuticals in United States wastewater effluent. *Environ Pollut* 121:199-205.

Huggett DB, Cook JC, Ericson JF, Williams RT. 2003b. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. *Hum Ecol Risk Assess* 9:1789-1799.

Huggett DB, Benson WH, Chipman K, Cook JC, Gray LE, Kinter LB, Meyerhoff RD, Trudeau VL. 2005. Role of mammalian data in determining pharmaceutical responses in aquatic species. In: Williams RT, editor. *Human pharmaceuticals: Assessing the impacts on aquatic ecosystems*. Pensacola (FL): SETAC Press. p. 149-181.

Hummert K, Vetter W, Luckas B. 1995. Levels of alpha-HCH, and enantiomeric ratios of alpha-HCH in marine mammals from the northern hemisphere. *Chemosphere* 31:3489-3500.

Hutt AJ. 2002. The development of single-isomer molecules: why and how. *CNS Spectrums* 7(suppl 1):14-22.

Jensen KM, Korte JJ, Kahl MD, Pasha MS, Ankley GT. 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comp Biochem Phys C* 128:127-141.

Kallenborn R, Hühnerfuss H. 2001. *Chiral Environmental Pollutants, Trace Analysis and Ecotoxicology*. Berlin (Germany): Springer-Verlag. 209 p.

Kane AS, Salierno JD, Gipson GT, Molteno TCA, Hunter C. 2004. A video-based movement analysis system to quantify behavioral stress responses of fish. *Water Res* 38:3993-4001.

Khan IA, Thomas P. 1992. Stimulatory effects of serotonin on maturational gonadotropin release in the Atlantic croaker, *Micropogonias undulates*. *Gen Comp Endocrinol* 88:288-396.

Kiely T, Donaldson D, Grube A. 2004. Pesticides industry sales and usage, 2000 and 2001 market estimates. Washington (DC): U.S. EPA Office of Pesticide Programs, Biological and Economic Analysis Division. www.epa.gov/oppbead1/pestsales/.

Kim H, Jeong K, Lee S, Jung S. 2003. Molecular modeling of the chiral recognition of propranolol enantiomers by a β-cyclodextrin. *Bull Korean Chem Soc* 24:95-98.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environ Sci Technol* 36:1202-1211.

Kovatcheva A, Golbraith A, Oloff S, Feng J, Zheng W, Tropsha A. 2005. QSAR modeling of datasets with enantioselective compounds using chirality sensitive molecular descriptors. *SAR QSAR Environ Res* 16(1-2):93-102.

Laurent P, Holmgren S, Nilsson S. 1983. Nervous and humoral control of the fish heart: structure and function. *Comp Biochem Physiol* 76:525-539.

Levin W, Buening MK, Wood AW, Chang RL, Kedzierski B, Thakker DR, Boyd DR, Gadaginamath GS, Armstrong RN, Yagi H, Karle JM, Slagal TJ, Jerina DM, Conney AH. 1980. An enantiomeric interaction in the metabolism and tumorgenicity of (+)- and (-)-benzo[*a*]pyrene 7,8-oxide. *J Biol Chem* 255(19):9067-9074.

Liebig M, Egeler P, Oehlmann J, Knacker T. 2005. Bioaccumulation of ^{14}C-17α-ethinylestradiol by the aquatic oligochaete *Lumbriculus variegatus* in spiked artificial sediment. *Chemosphere* 59:271-280.

Lilius H, Hästbacka T, Isomaa B. 1995. A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. *Environ Toxicol Chem* 14:2085-2088.

Lydy M, Belden J, Wheelock C, Hammock B, Denton D. . Challenges in regulating pesticide mixtures. *Ecol Soc* [Internet]. 2004 [cited 2007 Mar 21]; 9(6):1. Available from: http://www.ecologyandsociety.org/vol9/iss6/art1

Mandrioli R, Forti GC, Raggi MA. 2006. Fluoxetine metabolism and pharmacological interactions: The role of cytochrome P450. *Curr Drug Metab* 7:127-133.

Maruya KA, Smalling KL, Vetter W. 2005. Temperature and congener structure affect the enantioselectivity of toxaphene elimination by fish. *Environ Sci Technol* 39(11):3999-4004.

Mathieu A, Lemaire P, Carriere S, Drai P, Giudicelli J, Lafaurie M. 1991. Seasonal and sex-linked variations in hepatic and extrahepatic biotransformation activities in striped mullet (*Mullus barabatus*). *Ecotox Envioron Safe* 22:45-57.

Mathison IW, Solomons WE, Morgan PH, Tidwell RR. 1989. Structural features and pharmacologic activity. In: Foye WO, editor. *Principles of Medicinal Chemistry*. Philadelphia (PA): Lea & Febiger. p 49-77.

McConathy J, Owens MJ. 2003. Stereochemistry in drug action. *Primary Care Companion J Clin Psychiatry* 5(2):70-73.

Mehvar R, Brocks DR. 2001. Stereospecific pharmacokinetics and pharmacodynamics of beta-adrenergic blockers in humans. *J Pharm Pharm Sci* 4:185-200.

Metcalfe CD, Miao XS, Koenig BG, Struger J. 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower great lakes, Canada. *Environ Toxicol Chem* 22(12):2881-2889.

Mimeault C, Woodhouse AJ, Miao XS, Metcalfe CD, Moon TW, Trudeau VL. 2005. The human lipid regulator gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. *Aquat Toxicol* 73:44-54.

Mount DI, Brungs WA. 1967. A simplified dosing apparatus for fish toxicological studies. *Water Res* 1:21-29.

Nickerson JG, Dugan SG, Drouin G, Perry SF, Moon TW. 2003. Activity of the unique β-adrenergic Na^+/H^+ exchanger in trout erythrocytes is controlled by a novel β_3-AR subtype. *Am J Physiol Regul Integr Comp Physiol* 285:R2526-R535.

Nikolai LN, McCLure EL, MacLeod SL, Wong CS. 2006. Stereoisomer quantification of the β-blocker drugs atenolol, metoprolol, and propranolol in wastewaters by chiral high-performance liquid chromatography–tandem mass spectrometry. *J Chromatogr A* 1131:103-109.

Oaks JL, Gilbert M, Virani MZ, Watson RT, Meteyer CU, Rideout BA, Shivaprasad HL, Ahmed S, Chaudhry MJI, Arshad M, Mahmood S, Ali A, Khan AA. 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* 427:630-633.

[OECD] Organization for Economic Co-operation and Development. 1998. OECD Guidelines for testing of chemicals, *Daphnia magna* Reproduction Test, Test Method 211. Paris (France).

Padma TV, Dickhut RM, Ducklow H. 2003. Variations in α-Hexachlorocyclohexane enantiomer ratios in relation to microbial activity in a temperate estuary. *Environ Toxicol Chem* 22:1421-1427.

Perreault HAN, Semsar K, Godwin J. 2003. Fluoxetine treatment decreases territorial aggression in a coral reef fish. *Physiol Behav* 79:719-724.

Pham-Huy C, Radenen B, Sahui-Gnassi A, Claude JR. 1995. High-performance liquid chromatographic determination of (S)- and I-propranolol in human plasma and urine with a chiral β-cyclodextrin bonded phase. *J Chromatogr B* 665:125-132.

Postmes TJ, Prick R, Borens I. 1989. The deceleration of the heart frequency in the waterflea *Daphnia magna* by adrenoceptor agonists and antagonists. *Hydrobiologia* 171:141-148.

Ram JL, Crawford GW, Walker JU, Mojares JJ, Patel N, Fong PP, Kyozuka K. 1993. Spawning in the zebra mussel (*Dreissena polymorpha*): Activation by internal or external application of serotonin. *J Exp Zool* 265(5):587-598.

Sadahl JF, Baldwin DH, Jenkins JJ, Scholz NL. 2005. Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in Coho salmon exposed to chlorpyrifos. *Environ Toxicol Chem* 24(1):136-145.

Sanderson H, Johnson DJ, Wilson CJ, Brain RA, Solomon KR. 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. *Toxicol Lett* 144(3):383-395.

Seiler JP. 2002. Pharmacodynamic activity of drugs and ecotoxicology – can the two be connected? *Toxicol Lett* 131:105-115.

Shishkina GT, Dyalgo NN, Yudina AM, Kalinina TS, Tolstikova TG, Sorokina IV, Kovalenko IL, Anikina LV. 2006. The effects of fluoxetine and its complexes with glycerrhizic acid on behavior in rats and brain monoamine levels. *Neuroscience and Behavioral Physiology* 36(4):329-333.

Stanley JK, Ramirez AJ, Chambliss CK, Brooks BW. *Accepted with revision*. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. *Chemosphere*.

Stanley JK, Ramirez AJ, Mottaleb M, Chambliss CK, Brooks BW. 2006. Enantiospecific toxicity of the β-blocker propranolol to *Daphnia magna* and *Pimephales promelas*. *Environ Toxicol Chem* 25:1780-1786.

Stegeman JJ, Cehvion M. 1980. Sex differences in cytochrome P-450 and mixed function oxygenase activity in gonadally mature trout. *Biochem Pharmacol* 29:553-558.

Stegeman JJ, Pajor AM, Thomas P. 1982. Influence of estradiol and testosterone on cytochrome P-450 and monoxygenase activity in immature brook trout, *Salvelinus fontinalis*. *Biochem Pharmacol* 31(24):3979-3989.

Sweetman S, editor. 2006. *Martindale: The complete drug reference* [Internet]. London (UK): Pharmaceutical Press; [cited 2007 Mar 21]. Available from: ttp://www.medicinescomplete.com/mc/

Ternes TA. 1998. Occurrence of drugs in German sewage treatment plants and rivers. *Wat Res* 32:3245-3260.

Thomas KV, Hilton MJ. 2004. The occurrence of selected human pharmaceutical compounds in UK estuaries. *Mar Pollut Bull* 49:436-444.

Tran CD, Oliveira D, Grishko VI. 2004. Determination of enantiomeric compositions of pharmaceutical products by near-infrared spectrometry. *Anal Biochem* 325:206-214.

Tsai CL, Wang LH. 1997. Effects of thermal acclimation on the neurotransmitters serotonin and norepinephrine in the discrete brain of male and female tilapia, *Oreochromis mossambicus*. *Neurosci Lett* 233:77-80.

Tsujii S, Bray GA. 1998. A β-3 adrenergic agonist (BRL-37,344) decreases food intake. *Physiol Behav* 63:723-728.

[U.S. EPA] U.S. Environmental Protection Agency. 1992. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Washington (DC): EPA-821-R-02-012.

[U.S. EPA] U.S. Environmental Protection Agency. 1996. Ecological Effects Test Guidelines, OPPTS 850.1300, Daphnid Chronic Toxicity Test. Washington (DC): EPA 712–C–96–120.

[U.S. EPA] U.S. Environmental Protection Agency. 1998. Fate, transport and transformation test guidelines. Washington (DC): OPPTS 835.3300 Soil biodegradation.

[U.S. EPA] U.S. Environmental Protection Agency, Environmental Fate and Effects Division. 2000. Interim Policy for Stereoisomeric Pesticides. Washington (DC): www.epa.gov/oppefed1/ecorisk_ders/stereoisomer_policy.htm.

[U.S. EPA] U.S. Environmental Protection Agency. 2002a. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Washington (DC): EPA-821-R-02-012.

[U.S. EPA] U.S. Environmental Protection Agency. 2002b. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Washington (DC): EPA-821-R-02-013.

[U.S. FDA] U.S. Food and Drug Administration [Internet]. 1992 [cited 2007 Mar 21]. FDA's policy statement for the development of new stereoisomeric drugs. Available from: http://www.fda.gov/cder/guidance/.

[U.S. FDA] U.S. Food and Drug Administration [Internet]. 1998 [cited 2007 Mar 21]. . Guidance for industry, environmental assessment of human drug and biologics applications. Available from: http://www.fda.gov/cder/guidance/.

Uhler GC, Huminski PT, Les FT, Fong PP. 2000. Cilia-driven rotational behavior in gastropod (*Physa elliptica*) embryos induced by serotonin and putative serotonin reuptake inhibitors (SSRIs). *J Exp Zool* 286:414-421.

Vanderford BJ, Pearson RA, Rexing DJ, Snyder SA. 2003. Analysis of endocrine disruptors, pharmaceuticals, and personal care products in water using liquid chromatography/tandem mass spectrometry. *Anal Chem* 75:6265-6274.

Villegas-Navarro A, Rosas-L E, Reyes J. 2003. The heart of *Daphnia magna*: effect of four cardioactive drugs. *Comp Biochem Physiol C* 136:127-134.

Wedyan M, Preston MR. 2005. Isomer-selective adsorption of amino acids by components of natural sediment. *Environ Sci Technol* 39(7):2115-2119.

Weis JS, Smith G, Zhou T, Santiago-Bass C, Weis P. 2001. Effects of contaminants on behavior: biochemical mechanisms and ecological consequences. *BioScience* 51(3):209-217.

Weston JJ, Huggett DB, Rimoldi J, Foran CM, Slattery M. 2001. Determination of fluoxetine (Prozac^{TM}) and norfluoxetine in the aquatic environment. Annual meeting of the Society of Environmental Toxicology and Chemistry, Baltimore (MD).

Winberg S, Nilsson GE, Spruijt BM, Höglund U. 1993. Spontaneous locomotor activity of arctic charr measured by a computerized imaging technique: role of brain serotonergic activity. *J Exp Biol* 179:213-232.

Wong CS. 2006. Environmental fate processes and biochemical transformations of chiral emerging organic pollutants. *Anal Bioanal Chem* 386:544-558.

Wong DT, Reid LR, Threlkeld PG. 1988. Suppression of food intake in rats by fluoxetine: comparison of enantiomers and effects of serotonin antagonists. *Pharmacol Biochem Be* 31:475-479.

Wong DT, Bymaster FP, Reid LR, Mayle DA, Krushinski JH, Robertson DW. 1993. Norfluoxetine enantiomers as inhibitors of serotonin uptake in rat brain. *Neuropsychopharmacol* 8(4):337-344.